(51) Internationale Patentklassifikation [7] : **A61K 38/37, 38/36, 38/48, A61P 7/04 //** **(A61K 38/37, 38:17, 38:57, 38:49, 38:40,** **38:16)**	**A3**	(11) Internationale Veröffentlichungsnummer: **WO 00/27425** (43) Internationales Veröffentlichungsdatum: 18. Mai 2000 (18.05.00)

(54) Title: PHARMACEUTICAL PREPARATION CONTAINING A RECEPTOR–ANTAGONIST FOR TREATING
BLOOD–CLOTTING DISORDERS

(54) Bezeichnung: PHARMAZEUTISCHE PRÄPARATION, ENTHALTEND EINEN REZEPTOR–ANTAGONISTEN ZUR BEHAND-
LUNG VON BLUTGERINNUNGSSTÖRUNGEN

(57) Abstract

The invention relates to a pharmaceutical preparation for treating blood–clotting disorders which is characterized in that it contains
at least one protein selected from the group of pro–proteins of the blood clotting and additionally contains a coagulation–physiologically
inert receptor binding competitor. The invention also relates to the medical application of said preparation.

(57) Zusammenfassung

Beschrieben wird eine pharmazeutische Präparation zur Behandlung von Blutgerinnungsstörungen, die dadurch gekennzeichnet ist,
daß sie mindestens ein Protein, ausgesucht aus der Gruppe von pro–Proteinen der Blutgerinnung und weiters einen gerinnungsphysiologisch
inerten Rezeptor–Bindungskompetitor enthält, sowie deren medizinische Verwendung.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   A61K38/37   A61K38/36   A61K38/48   A61P7/04   //(A61K38/37, 38:17,38:57,38:49,38:40,38:16)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 34930 A (EIBL JOHANN ; IMMUNO AG (AT); MITTERER ARTUR (AT); DORNER FRIEDRICH) 25 September 1997 (1997-09-25) abstract; claims 1,16,18 page 1, line 1 -page 3, line 11 ---	1-5,9, 12,13, 15-17, 19,20, 22,23,25
X	EP 0 519 900 A (IMMUNO AG) 23 December 1992 (1992-12-23) abstract; claims 1-3 page 2, left-hand column, line 1 -right-hand column, line 41 --- -/--	1,2,4,5, 9,12,13, 15,17,20

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 May 2000	13/06/2000

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 32459 A (STRINGER KATHLEEN A ;REPINE JOHN E (US); MCCORD JOE M (US); HYBERT) 30 July 1998 (1998-07-30) page 3, line 28 — line 29 page 14, line 16 —page 15, line 2; claims 1,13	14
X	WO 95 25536 A (UNIV WASHINGTON ;JEWISH HOSPITAL OF ST LOUIS (US); SCHWARTZ ALAN L) 28 September 1995 (1995-09-28) page 2, line 20 —page 3, line 31; claims 1-3,7-9,11	23
X	WO 94 14471 A (UNIV WASHINGTON) 7 July 1994 (1994-07-07) abstract; claim 1 page 13, line 12 —page 14, line 23 page 15, line 1 — line 22	23
A	NEELS ET AL: "ligand receptor interaction of the low density lipoprotein receptor-related protein, a multi-ligand endocytic receptor" FIBRINOLYSIS & PROTEOLYSIS, vol. 12, no. 4, 1998, pages 219-240, XP000907114 abstract page 220, left-hand column, line 48 —page 222, right-hand column, line 50; table 1 page 227, left-hand column, line 1 —page 231, right-hand column, line 5	13
A	NOORMAN F ET AL: "regulation of tissue-type plasminogen activator concentration by clearance via the mannose receptor and other receptors" FIBRINOLYSIS & PROTEOLYSIS, vol. 11, no. 4, 1997, pages 173-186, XP000907181 abstract page 174, left-hand column, line 16 —right-hand column, line 54 page 175, left-hand column, line 43 —right-hand column, line 47 page 176; table 1 page 177, right-hand column, line 7 —page 178, left-hand column, line 17 page 180, left-hand column, line 13 —right-hand column, line 54	13

—/—

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Im Recherchenbericht angeführtes Pat ntdokument		Datum der Veröffentlichung	Mitglied(r) der Patentfamilie		Datum der Veröffentlichung
WO 9734930	A	25-09-1997	AT	403764 B	25-05-1998
			AT	49496 A	15-10-1997
			CA	2248960 A	25-09-1997
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EP 0519900	A	23-12-1992	AT	402263 B	25-03-1997
			AT	124091 A	15-08-1996
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			AU	1830492 A	24-12-1992
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			JP	5170665 A	09-07-1993
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WO 9525536	A	28-09-1995	US	5650391 A	22-07-1997
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WO 9414471	A	07-07-1994	US	5474766 A	12-12-1995
			AU	5955494 A	19-07-1994

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	YAKYAEV ET ALL: "cellular uptake and degradation of the thrombin activated factor VIII fragmets" BLOOD, vol. 90, no. 10, suppl 1, - 1997 page 31a XP000907187 cited in the application the whole document	1-25
A	NARITA MASAAKI ET AL: "The low-density lipoprotein receptor-related protein (LRP) mediates clearance of coagulation factor Xa in vivo." BLOOD JAN. 15, 1998, vol. 91, no. 2, 15 January 1998 (1998-01-15), pages 555-560, XP002137878 ISSN: 0006-4971 the whole document	1-25
A	KOUNNAS MARIA Z ET AL: "Cellular internalization and degradation of antithrombin III-thrombin, heparin cofactor II-thrombin, and alpha-1-antitrypsin-trypsin complexes is mediated by the low density lipoprotein receptor-related protein." JOURNAL OF BIOLOGICAL CHEMISTRY 1996, vol. 271, no. 11, 1996, pages 6523-6529, XP002137879 ISSN: 0021-9258 the whole document	1-25
P,X	SCHWARZ, HANS PETER ET AL: "Involvement of low-density lipoprotein receptor - related protein (LRP) in the clearance of factor VIII in von Willebrand factor -deficient mice" BLOOD (2000), 95(5), 1703-1708 , XP002137880 the whole document	1-7,9, 12,13, 15-17, 19,20, 22,23,25

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/AT 99/00271

Box I   **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐   Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒   Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

   ## See supplemental sheet Additional Matter PCT/ISA/210

3. ☐   Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐   As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐   As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐   As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐   No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐   The additional search fees were accompanied by the applicant's protest.

           ☐   No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

Continuation of Field I.2

Relevant Patent Claims Nos. 1-4, 7, 9, 12, 15-19 (Only Claim No. 8 can be dependent on
Claim No. 7 since no reference to a mixture is given in Claims Nos. 1-6. Therefore, the
search was directed at all of Claim No. 8.) relate to a compound which is respectively
characterized by a desired feature, namely "receptor binding competitor".
(Only Claim No. 8 can be dependent on Claim No. 7 since no reference to a mixture is given
in Claims Nos. 1-6. Therefore, the search was directed at all of Claim No. 8.)

The patent claims thus comprise all products, etc. which comprise this feature, whereas only a
limited number of such products, etc. are supported by the description as per the patent
application under the terms of PCT Article 5. In the case in question, the patent claims lack
the corresponding support and the patent application lacks the necessary disclosure to such a
degree that a meaningful search appears to be impossible to conduct with respect to the entire
scope for which protection is sought.

For this reason, the search was directed at the sections of the patent claims which can be
regarded as clear, supported or disclosed in the above-mentioned sense, namely to the sections
relating to the LRP or mannose receptor binding competitor.

The applicant is therefore advised that patent claims or sections of patent claims laid to
inventions for which no international search report was drafted normally cannot be the subject
of an international preliminary examination (PCT Rule 66.1(e)). Similar to the authority
entrusted with the task of carrying out the international preliminary examination, the EPO
also does not generally carry out a preliminary examination of subject matter for which no
search has been conducted. This is also valid in the case when the patent claims have been
amended after receipt of the international search report (PCT Article 19), or in the case when
the applicant submits new patent claims pursuant to the procedure in accordance with PCT
Chapter II.

INTERNATIONALER RECHERCHENBERICHT

Feld I   Bemerkungen zu den Ansprüchen, die sich als nicht recherchierbar erwiesen haben (Fortsetzung von Punkt 2 auf Blatt 1)

Gemäß Artikel 17(2)a) wurde aus folgenden Gründen für bestimmte Ansprüche kein Recherchenbericht erstellt:

1. ☐ Ansprüche Nr.
weil sie sich auf Gegenstände beziehen, zu deren Recherche die Behörde nicht verpflichtet ist, nämlich

2. ☒ Ansprüche Nr. —
weil sie sich auf Teile der internationalen Anmeldung beziehen, die den vorgeschriebenen Anforderungen so wenig entsprechen,
daß eine sinnvolle internationale Recherche nicht durchgeführt werden kann, nämlich
siehe Zusatzblatt WEITERE ANGABEN PCT/ISA/210

3. ☐ Ansprüche Nr.
weil es sich dabei um abhängige Ansprüche handelt, die nicht entsprechend Satz 2 und 3 der Regel 6.4 a) abgefaßt sind.

Feld II   Bemerkungen bei mangelnder Einheitlichkeit der Erfindung (Fortsetzung von Punkt 3 auf Blatt 1)

Die internationale Recherchenbehörde hat festgestellt, daß diese internationale Anmeldung mehrere Erfindungen enthält:

1. ☐ Da der Anmelder alle erforderlichen zusätzlichen Recherchengebühren rechtzeitig entrichtet hat, erstreckt sich dieser
internationale Recherchenbericht auf alle recherchierbaren Ansprüche.

2. ☐ Da für alle recherchierbaren Ansprüche die Recherche ohne einen Arbeitsaufwand durchgeführt werden konnte, der eine
zusätzliche Recherchengebühr gerechtfertigt hätte, hat die Behörde nicht zur Zahlung einer solchen Gebühr aufgefordert.

3. ☐ Da der Anmelder nur einige der erforderlichen zusätzlichen Recherchengebühren rechtzeitig entrichtet hat, erstreckt sich dieser
internationale Recherchenbericht nur auf die Ansprüche, für die Gebühren entrichtet worden sind, nämlich auf die
Ansprüche Nr.

4. ☐ Der Anmelder hat die erforderlichen zusätzlichen Recherchengebühren nicht rechtzeitig entrichtet. Der internationale Recher-
chenbericht beschränkt sich daher auf die in den Ansprüchen zuerst erwähnte Erfindung; diese ist in folgenden Ansprüchen er-
faßt:

Bemerkungen hinsichtlich eines Widerspruch

☐ Di zusätzlichen G bühren wurden vom Anmelder unter Widerspruch gezahlt.

☐ Die Zahlung zusätzlicher Recherchengebühren erfolgte ohn Widerspruch.

Formblatt PCT/ISA/210 (Fortsetzung von Blatt 1 (1))(Juli 1998)

Fortsetzung von Feld I.2

Die geltenden Patentansprüche 1-4, 7, 9, 12, 15-19 (Da es keine Referenz zu einem gemisch in den Ansprüchen 1-6 gibt, kann nur Anspruch 8 vom Anspruch 7 abhängig sein. Deswegen wurde die Recherche auf den ganzen Anspruch 8 gerichtet) beziehen sich auf eine Verbindung, jeweils charakterisiert durch eine erstrebenswerte Eigenschaft, nämlich "Rezeptor-Bindungskompetitor".
(Da es keine Referenz zu einem gemisch in den Ansprüchen 1-6 gibt, kann nur Anspruch 8 vom Anspruch 7 abhängig sein. Deswegen wurde die Recherche von dem ganzen Anspruch 8 gerichtet.)

Die Patentansprüche umfassen daher alle Produkte etc., die diese Eigenschaft aufweisen, wohingegen die Patentanmeldung Stütze durch die Beschreibung im Sinne von Art. 5 PCT nur für eine begrenzte Zahl solcher Produkte etc. liefert. Im vorliegenden Fall fehlen den Patentansprüchen die entsprechende Stütze bzw. der Patentanmeldung die nötige Offenbarung in einem solchen Maße, daß eine sinnvolle Recherche über den gesamten erstrebten Schutzbereich unmöglich erscheint.

Daher wurde die Recherche auf die Teile der Patentansprüche gerichtet, welche im o.a. Sinne als klar, gestützt oder offenbart erscheinen, nämlich die Teile betreffend die LRP oder Mannose-rezeptor Bindungkompetitor.

Der Anmelder wird darauf hingewiesen, daß Patentansprüche, oder Teile von Patentansprüchen, auf Erfindungen, für die kein internationaler Recherchenbericht erstellt wurde, normalerweise nicht Gegenstand einer internationalen vorläufigen Prüfung sein können (Regel 66.1(e) PCT). In seiner Eigenschaft als mit der internationalen vorläufigen Prüfung beauftragte Behörde wird das EPA also in der Regel keine vorläufige Prüfung für Gegenstände durchführen, zu denen keine Recherche vorliegt. Dies gilt auch für den Fall, daß die Patentansprüche nach Erhalt des internationalen Recherchenberichtes geändert wurden (Art. 19 PCT), oder für den Fall, daß der Anmelder im Zuge des Verfahrens gemäß Kapitel II PCT neue Patentanprüche vorlegt.

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**Pharmazeutische Präparation enthaltend einen Rezeptor-
<u>Antagonisten zur Behandlung von Blutgerinnungsstörungen</u>**

Die Erfindung betrifft eine pharmazeutische Präparation auf Ba-
sis von Blutgerinnungsproteinen und Maßnahmen zur Verlängerung
deren biologischen Halbwertszeit.

Therapeutische Proteine, insbesondere Präparationen zur Behand-
lung von Blutgerinnungsstörungen, welche Blutgerinnungsproteine,
wie z.B. von Willebrand-Faktor (vWF), beinhalten, haben oft re-
lativ kurze Halbwertszeiten im Organismus. Dadurch kann eine
therapeutische Verabreichung solcher Präparationen innerhalb
kurzer Zeit wirkungslos werden oder zumindest in ihrer Wirksam-
keit stark beeinträchtigt werden.

Vieles an den physiologischen Abbauwegen von Blutgerinnungspro-
teinen ist ebenso unbekannt wie die genauen Faktoren, die deren
Halbwertszeit im Körper bestimmen.

So ist lediglich bekannt, daß aktivierter Faktor VIII über seine
A2-Domäne an das Lipoproteinrezeptor-verwandte Protein (lipopro-
tein receptor related protein; LRP) bindet, wobei diese Bindung
sowohl für die Internalisierung von Faktor VIII als auch für
dessen Abbau zuständig ist. Es ist auch bekannt, daß durch ein
Rezeptor-assoziiertes Protein (RAP), einem Inhibitor von LRP,
die Internalisierung der A2-Domäne von Faktor VIII gehemmt wer-
den kann, wobei angenommen wird, daß die Dissoziation der A2-
Untereinheit vom übrigen aktivierten Faktor VIII-Molekül rever-
sibel ist.

Das "low density lipoprotein related protein" ist ein multi-
funktionell endozytotisch aktiver Rezeptor, der strukturell wie
funktionell unterschiedliche Liganden bindet und endozytieren
kann. RAP ("receptor-associated protein") inhibiert alle Inter-
aktionen der Liganden mit LRP in vitro.

Prinzipiell wird vermutet, daß dieser Mechanismus von Bedeutung
bei der Aktivitätsregulierung von Faktor VIIIa sein könnte
(Blood 90 (10) Suppl. 1: 31a (1997)).

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Es ist auch bekannt, daß LRP als Abbaurezeptor (Clearence receptor) für Gewebeplasminogenaktivator (tPA) fungieren kann, d.h. für dessen Entfernung aus der Blutzirkulation verantwortlich ist. Weiters scheinen dabei auch weitere Rezeptoren, wie z.B. der Mannose-Rezeptor, von Bedeutung zu sein. Andererseits ist auch bekannt, daß LRP bei der Clearence von vielen verschiedenen Liganden, wie Proteinasen, Inhibitoren, deren Komplexe mit Proteinasen, sowie verschiedenen Lipoproteinen, beteiligt sein kann. LRP kann sowohl zellgebunden vorliegen als auch als lösliche Form (Quinn K.A. et al., XVIIth Congress of the International Society of Thrombosis and Haemostasis, 1999, Abstract).

Es wurde gefunden, daß tPA eine sehr schnelle Clearence im Blutkreislauf erfährt, und daß Mutanten von tPA sowie Inhibitoren der Clearence dazu verwendet werden können, um die Dosierung von tPA bei der Thrombolysetherapie zu verringern (Fibrinolysis and Proteolysis (1997), S. 173-186).

In RAP-defizienten Mäusen konnte nachgewiesen werden, daß die Clearence von α_2-Makroglobulin in der Leber gestört ist. Hierbei wurde auch nachgewiesen, daß die Menge von reifem prozessierten LRP sowohl in der Leber als auch im Gehirn reduziert ist (PNAS, 92 (1995), S. 4537-4541).

In der EP-0 713 881-A2 werden von Willebrand-Faktor (vWF)-Konzentrate in Kombination mit Antithrombolytika und/oder Fibrinolytika beschrieben. Zweck dieser Präparate ist die Verringerung der Blutungsrisiken bei einer Antikoagulationstherapie.

In der US 5 304 383 wird eine pharmazeutische Präparation, enthaltend lys-Plasminogen in Kombination mit einem Serinproteaseinhibitor, wie Aprotinin oder α_2-Makroglobulin, beschrieben, welche zur Behandlung von Plasminogen-Mangel und Thrombosen eingesetzt werden kann.

Die Erfindung stellt sich zur Aufgabe, eine pharmazeutische Präparation zur Verfügung zu stellen, auf Basis von einem Blutgerinnungsprotein, welches sich durch eine verbesserte in vivo-Halbwertszeit auszeichnet. Die Erfindung stellt sich weiters zur

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Aufgabe, die in vivo Halbwertszeit eines Blutgerinnungsproteins
zu verlängern, um einerseits den endogenen Gehalt an dem Blutge-
rinnungsprotein zu stabilisieren und andererseits die Effizienz
von einem exogenen Blutgerinnungsprotein zu steigern.

Die Aufgabe wird erfindungsgemäß durch eine pharmazeutische Prä-
paration zur Behandlung von Blutgerinnungsstörungen gelöst, wel-
che Präparation mindestens ein Protein, ausgesucht aus der
Gruppe von einem pro-Protein der Blutgerinnung enthält. Diese
pharmazeutische Präparation ist weiters durch einen Gehalt an
einem Rezeptor-Bindungskompetitor gekennzeichnet, welcher al-
leine gerinnungsphysiologisch inert ist, wie beispielsweise Li-
poproteine. Dadurch wird die Wirkung des Proteins auf die Blut-
gerinnung nicht unmittelbar beeinflußt, der Rezeptor-Bindungs-
kompetitor trägt lediglich zur Stabilisierung des Proteins in
vivo bei.

Das Protein in der erfindungsgemäßen Präparation ist vorzugs-
weise ein Blutgerinnungsprotein, ausgesucht aus der Gruppe von
Faktor II, V, VII, VIII, IX, X, XI, XII, vWF und Protein C.
Diese Proteine sind die Proformen von der gerinnungsphysiolo-
gisch wirksamen aktiven Form. Beispielsweise ist Faktor II (Pro-
thrombin) die Proform des gerinnungsaktiven Thrombins, welches
Fibrinogen zu Fibrin enzymatisch spaltet und damit ein Blutge-
rinnsel hervorruft. Zu diesen Proteinen zählen einerseits die
Vitamin K-abhängigen Proteine, welche auch in einem Prothrombin-
komplex-Präparat auf Basis der Faktoren II, IX und X und gegebe-
nenfalls Faktor VII und Protein C vorkommen. Weiters ist ein
mögliches Protein in der erfindungsgemäßen Präparation des vWF
bzw. dessen analoge Proteine. vWF zeigt vor allem in Plättchen-
reichem Plasma seine Eigenständigkeit als Blutgerinnungsfaktor,
unabhängig von seiner Rolle als Träger und Stabilisator des Fak-
tor VIII (Beguin et al., Thrombosis and Haemostasis 78 (1), 590-
594 (1997)). vWF wird beispielsweise aus Plasma oder einer Plas-
mafraktion gewonnen, kann aber auch durch rekombinante DNA-Tech-
nologie, durch Expression einer transformierten Zelle, herge-
stellt werden. Beispielsweise wird ein rekombinanter vWF nach
der Vorschrift von Pannekoek (EP 0 197 592) bzw. Fischer et al.
(WO 96/10584) hergestellt.

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Auch die Gruppe der Blutgerinnungszymogene bzw. Vorstufen der
gerinnungsphysiologisch aktiven Blutgerinnungproteine sind ei-
nerseits aus Plasma oder einer Plasmafraktion erhältlich und
entsprechen den nativen Proteinen. Andererseits können die nati-
ven Proteine bzw. deren Derivate auch aus Zellkulturüberständen
gewonnen werden. Die Zellen werden dabei vorzugsweise durch re-
kombinante DNA-Technologie transformiert, um die rekombinanten
Formen der Blutgerinnungsproteine zu erhalten.

Mutanten bzw. Analoge sind insoweit in der erfindungsgemäßen
Präparation enthalten, wie diese noch zur Behandlung von Blutge-
rinnungsstörungen eingesetzt werden können und an einen Rezeptor
funktionell, also kompetitiv, binden können.

Als Rezeptor für die Proteine der erfindungsgemäßen Präparation
ist vor allem der Lipoproteinrezeptor zu nennen bzw. LRP. Ein
weiterer Rezeptor, an dem die Proteine binden, ist beispiels-
weise der Mannose-Rezeptor. Es hat sich erfindungsgemäß überra-
schenderweise herausgestellt, daß die Liganden des LRP vor allem
für pro-Proteine der Blutgerinnung eine positive Wirkung in vivo
zeigen, indem diese Liganden an den Rezeptor kompetitiv binden.
Dies war insofern überraschend, da nicht vermutet werden konnte,
daß pro-Proteine der Blutgerinnung und vWF an LRP binden können.
LRP war vor allem zur Bindung von Enzymen bzw. deren Komplexe
mit Inhibitoren bekannt. Durch die kompetitive Eigenschaft der
Bindung an LRP wird die vorzeitige Bindung des Proteins in der
pharmazeutischen Präparation bzw. des endogenen Blutgerinnungs-
zymogens und vWF verhindert, wodurch dessen biologische Verfüg-
barkeit wesentlich verbessert wird.

Als Rezeptor-Bindungskompetitor können eine Reihe von Proteinen
eingesetzt werden, wobei diejenigen bevorzugt sind, deren phy-
siologische Toleranz geprüft ist.

Erfindungsgemäß bevorzugt ist der Rezeptor-Bindungskompetitor
ausgewählt aus der Gruppe der Enzyme, insbesondere Tissue-type
Plasminogenaktivator (tPA), Urokinase (u-PA), pro-Urokinase
(pro-u-PA), Lipoproteinlipase (LPL) und Kallikrein; Inhibitoren,
insbesondere Plasminogen-Aktivator-Inhibitor-1 (PAI-1), Tissue
factor pathway Inhibitor (TFPI) und Aprotinin; Enzym-Inhibitor-

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Komplexe, insbesondere t-PA-PAI-1, u-PA-PAI-1 und Thrombin-PAI-1, α_2-Macroglobulin-Proteinase (fast form) α_2M, α_2M', Pregnancy zone protein-Proteinase, Elastase-α^1-Antitrypsin, Thrombin-Antithrombin III, Thrombin-Heparin-Cofactor II und u-PA-Protease-Nexin I; Lipoproteine, insbesondere low density-Lipoprotein (LDL), Apolipoprotein E angereichertes β-very low density-Lipoprotein, (apo E-β-VLDL), LPL angereichertes VLDL (LPL-VLDL) und LPL angereichertes β-VLDL (LPL-β-VLDL); Matrix-Proteine, insbesondere Thrombospondin 1 und 2; Toxine und Viren, insbesondere Pseudomonas Exotoxin A, und Minor-group human Rhinovirus; oder andere Liganden, insbesondere Apolipoprotein E (apo E), Lactoferrin, Rezeptor-assoziiertes Protein (RAP), FVIII und vWF.

Der kompetitiv wirkende Ligand ist vorzugsweise ausgewählt unter den physiologischen Proteinen, die vorzugsweise in der Zirkulation als (humanphysiologische) extrazelluläre Proteine auftreten. Diese können in nativer Form eingesetzt werden, oder als deren Analoge mit der Eigenschaft der Bindung an den Rezeptor.

Bevorzugterweise handelt es sich bei den kompetitiv wirkenden Liganden um RAP oder ein Derivat davon. Besonders bevorzugt ist ein Fragment von RAP, das jene Domäne umfaßt, die die Bindung von Proteinen an LRP verhindert und dadurch die Internalisierung und Clearance der Proteine inhibiert (I. Warshawsky et al., J. Biol.Chem., 1993, vol. 15, pp. 22046-22054; Bu G., EMBO J., 1995, vol. 14, pp. 2269-2280). Es kann sich dabei um RAP oder ein Derivat davon handeln, das humanen Ursprungs ist, aber auch um tierisches RAP oder ein Derivat davon, beispielsweise aus Mäusen, Ratten oder anderen Tieren.

Um die Wirkung der pharmazeutischen Präparation bzw. der endogenen Proteine nicht zu beeinträchtigen, ist der Rezeptor-Bindungskompetitor ausgewählt unter den gerinnungsphysiologisch inerten Proteinen, darunter beispielsweise "low density lipoprotein" (LDL) und Apolipoprotein.

Als gerinnungsphysiologisch inerter Rezeptor-Bindungskompetitor können auch Gemische dieser Rezeptor-Bindungskompetitoren eingesetzt werden, die eventuell nicht als einzelne Proteine, jedenfalls aber im Gemisch keinen unerwünschten Einfluß auf das Ge-

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rinnungssystem ausüben. Beispiele für solche Gemische sind
Thrombin mit ATIII oder mit Heparin-Cofaktor II, oder mit Plas-
minogen-Aktivator-Inhibitor-1 (PAI-1), weiters auch
Elastase und α_1 -Antitrypsin, tPA mit PAI-1. Beispielsweise ist
ein bevorzugtes Gemisch tPA und Aprotinin, welches sich hervor-
ragend zur Stabilisierung etwa des Faktor VIII in vivo eignet.
Der gewählte Rezeptor-Bindungskompetitor ist hier also ein Li-
gand, dessen gerinnungsphysiologische Wirkung unterbunden ist,
gegebenenfalls durch die Antagonisierung mit dem entsprechenden
Inhibitor.

Anstelle eines Gemisches aus einem prinzipiell zwar wirksamen
Rezeptor-Bindungskompetitor und einem geeigneten Antagonisten
kann auch eine mutierte Form des Rezeptor-Bindungskompetitors
verwendet werden, der durch geeignete Mutationen (z.B. im akti-
ven Zentrum) inaktiviert ist (dessen Affinität gegenüber dem Re-
zeptor aber nicht wesentlich verändert (verringert) ist.) Bei-
spiele hierfür sind u.a. mutierte Formen von tPA, Urokinase,
Kallikrein, Renin, Thrombin (WO 96/41868) etc.

Es war überraschend, daß rekombinanter tPA (rtPA) den endogenen
Faktor VIII-Gehalt in einem vWF-defizienten Hund über einen län-
geren Zeitraum stabilisierte, wodurch die Hämophilie behandelt
werden konnte. Obwohl der natürliche Stabilisator des Faktor
VIII, nämlich vWF, fehlte, konnte der LRP-Ligand bzw. das Ge-
misch der LRP-Liganden die Funktion des vWF übernehmen. Der Fak-
tor VIII-Gehalt wurde um ca. 50 % erhöht und blieb bei diesem
Niveau mehrere Tage. Das Enzym tPA ist ein bekannter Ligand des
LRP. Die Verabreichung des rtPA an ein Säugetier blockiert also
LRP und verhindert somit den metabolischen Abbau der Gerinnungs-
faktoren, wie z.B. Faktor VIII. Dieser blockierende Effekt wird
noch durch Aprotinin verstärkt. Der Effekt von Aprotinin alleine
konnte auch in einem vWF-defizienten Hund gezeigt werden, der
eine Kombination von rvWF und Aprotinin erhielt. Dadurch wurde
wiederum eine Erhöhung des Faktor VIII-Gehaltes über einen län-
geren Zeitraum bewirkt.

Ein weiterer bevorzugter Rezeptor-Bindungskompetitor ist Aproti-
nin, welches sich hervorragend zur Stabilisierung des vWF eig-
net. Aprotinin kann beispielsweise nicht nur in einer vWF-Präpa-

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ration zur verbesserten biologischen Verfügbarkeit beitragen,
sondern auch, als einzelne Wirksubstanz verabreicht, zu verbes-
serten Zustand eines vWF-defizienten Patienten beitragen.

In vielen Fällen wird ein Patient mit einem Mangel an einem be-
stimmten Protein dann mit dem jeweiligen Rezeptor-Bindungskompe-
titor behandelt, um die Bildung des endogenen Proteins zu ermög-
lichen bzw. das Protein zu stabilisieren. Dies ist bei Patienten
mit einem phänotypischen Gerinnungsproteinmangel möglich.

Das erfindungsgemäße Präparat bzw. die genannten Wirksubstanzen
werden vorzugsweise als behandelte Präparate bzw. Proteine ein-
gesetzt, um eine Übertragung von möglicherweise vorhandenen Pa-
thogenen, wie Viren oder Prionen (TBE), auszuschließen. Bei der
Gewinnung aus biologischen Materialien, wie Blut, Plasma, Plas-
mafraktionen oder Zellkulturen, existiert ein Risiko der Konta-
mination mit Humanpathogenen, das jedoch durch eine entspre-
chende Behandlung zur Inaktivierung bzw. Abreicherung eliminiert
werden kann. Zu den effektiven Maßnahmen zur Inaktivierung von
Viren zählen beispielsweise die Behandlung mit organischen Lö-
sungsmitteln und/oder Detergenzien (EP-0 131 740, EP-0 050 061,
virus inactivating eluent), die Behandlung mit chaotropen Mit-
teln (WO 90 15 613) Hitzebehandlungsverfahren, vorzugsweise in
lyophilisiertem, trockenem oder feuchtem Zustand (siehe EP-0 159
311), Kombinationsverfahren wie das der (EP-0 519901) und physi-
kalische Methoden. Letztere bewirken die Inaktivierung von Vi-
ren, beispielsweise durch Bestrahlung mit Licht, etwa in Gegen-
wart von Photosensibilisatoren (EP-0 471 794 und WO/AT
97/00068).

Zu dem Abreicherungsverfahren der Humanpathogene zählen insbe-
sondere die Filtrationen unter Verwendung von Ultrafiltern, Tie-
fenfiltern oder Nanofiltern (A 341/98). Aber auch Fällungs-
schritte bzw. andere Proteinreinigungsmaßnahmen, wie die der Ad-
sorption, tragen zur Abreicherung von möglicherweise vorhandenen
Pathogenen bei.

Die erfindungsgemäße Präparation kann einerseits als fertige
Formulierung, d.h. als Gemisch des Proteins mit dem Rezeptor-
Bindungskompetitor zur Verfügung gestellt werden. Andererseits

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ist es auch möglich, ein Set zur Verfügung zu stellen, welches
A) das Protein und B) den Rezeptor-Bindungskompetitor enthält.
Das Set hat den Vorteil, daß die Dosis der einzelnen Komponenten
variabel ist und die Form der Administration den vorliegenden
Verhältnissen angepaßt werden kann. Beispielsweise ist neben der
iv-Verabreichung auch die intramuskuläre, subkutane oder orale
Verabreichung der Wirkstoffe möglich. Die Wirksubstanzen können
gemeinsam, also simultan, aber auch parallel oder konsekutiv dem
Patienten verabreicht werden. Bei Verwendung eines Sets ist die
erfindungsgemäße Präparation vorzugsweise in Fertigspritzen mit
den einzelnen Komponenten vorgefertigt.

Eine erfindungsgemäße Indikation für das Präparat ist beispiels-
weise eine Behandlung eines Patienten mit einem phänotypischen
Gerinnungsfaktormangel, z.B. eines vWF-defizienten Patienten.
Dabei wird erfindungsgemäß ein Präparat hergestellt auf Basis
des Gerinnungsfaktors, der im Patienten mangelhaft ist, mit der
erfindungsgemäßen Kombination des Rezeptor-Bindungskompetitors.
Aber auch der Rezeptor-Bindungskompetitor alleine entspricht der
erfindungsgemäßen Indikation. Beispielsweise wird bei einem Man-
gel an funktionellem Faktor VIII der Rezeptor-Bindungskompetitor
für Faktor VIII verabreicht, welcher eventuell das Gemisch tPA
und Aprotinin ist.

Gegenstand der vorliegenden Erfindung ist auch die Verwendung
einer LRP-Ligandenpräparation zur Herstellung eines Mittels zur
Behandlung von phänotypischem Gerinnungsfaktormangel (z.B. vWF-,
Faktor VIII- oder Faktor IX-Mangel) oder zur Verlängerung der
biologischen Halbwertszeit eines Proteins.

Die Erfindung wird an Hand der nachfolgenden Beispiele und der
Zeichnungen, auf die sie jedoch nicht beschränkt sein soll, nä-
her erläutert.

Es zeigen:

Fig. 1: Faktor VIII-Bestimmung im vWF-defizienten Hund

Fig. 2: vWF und Faktor VIII-Bestimmung in vWF-defizientem Hund

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B e i s p i e l e :

B e i s p i e l 1 :

Ein Kooiker Hund mit schwerem von Willebrand Faktor-Mangel vom
Typ 3 (Rieger et al., Thromb. Haemost. (1998), 80: 332-337)
männlichen Geschlechts, 8,5 kg Körpergewicht, 2 Jahre alt, wurde
mit 10 mg/kg Körpergewicht Ketamin (Ketasol, Dr. E. Gräub AG,
Bern, Schweiz) und 1 mg/kg Xylaxin (Xylasol, Dr. E. Gräub AG,
Bern, Schweiz) narkotisiert. Anschließend wurde ein permanenter
venöser Zugang über einen Unterarm geschaffen und über eine Ka-
nüle Aprotinin (Pantinol 100.000 K.I.E. Ampullen, Gerot Pharma-
zeutika, Wien) in einer Dosis von 10 000 K.I.E. pro kg Körperge-
wicht als Bolus verabreicht. Anschließend wurde rekombinanter
Gewebeplasminogenaktivator (Actilyse, Boehringer Mannheim)
(rtPA) gemäß Herstellerangabe mit destilliertem Wasser rekonsti-
tuiert und dem von Willebrand-defizienten Hund in einer Dosis
von 0,25 mg/kg Körpergewicht als Bolus verabreicht. Gleichzeitig
wurde eine Blutprobe auf Zitrat entnommen. Innerhalb der ersten
Stunde der Behandlung wurde eine Coinfusion von Aprotinin (3000
K.I.E. pro kg Körpergewicht) und rtPA 0,75 mg/kg Körpergewicht
verabreicht und nach einer Stunde wurde auf eine Infusion ge-
wechselt, die Aprotinin in einer Dosis von 3000 K.I.E. pro kg
Körpergewicht und rtPA von 0,5 mg/kg Körpergewicht enthielt, und
diese über eine Stunde verabreicht. Anschließend, d.h. nach zwei
Stunden, wurde über eine weitere Stunde Aprotinin in einer Dosis
von 3000 K.I.E./kg Körpergewicht infundiert. Zu den Zeitpunkten
30 min, 1, 3, 24, 48, 92 und 96 Stunden nach Versuchsbeginn wur-
den Blutproben auf Zitrat abgenommen und durch Zentrifugation
daraus plättchenarmes Plasma hergestellt und bei -20°C tiefge-
froren und bis zur weiteren Analyse gelagert.

In den gefrorenen Blutproben wurde nach Abschluß des Infusions-
experimentes Blutgerinnungsfaktor VIII mit zwei unterschiedli-
chen Bestimmungsmethoden quantitativ bestimmt. Erstens wurde die
Zweistufengerinnungsmethode gemäß der Methode von Austen, D.E.G.
und Rhymes, I.L., A Laboratory Manual of Blood Coagulation,
Oxford, UK, Blackwell Scientific, (1975), unter Verwendung der
Reagentien des Zweistufen-Faktor VIII - Test-Kits der IMMUNO AG,
Wien, verwendet. Zweitens wurde Faktor VIII mit dem chromogenen

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Faktor VIII-Test-Kit, Immunochrom FVIII:C der Immuno AG, Wien,
bestimmt (Lang H., Oberreiter M., Moritz, B. Thromb. Haemost.
65:943, (1991)). Faktor VIII wurde gegen einen humanen Plasma
Faktor VIII-Standard, der gegen den dritten internationalen
Standard 91/666 kalibriert worden war, gemessen. Die Faktor
VIII-Aktivität wird in humanen Faktor VIII-Einheiten pro ml aus-
gedrückt. Das Resultat dieser Bestimmung ist in der Fig. 1 zu-
sammengefaßt. In den beiden unabhängig voneinander durchgeführ-
ten Faktor VIII-Bestimmungsmethoden zeigte sich ein Anstieg der
Faktor VIII-Plasmaaktivität, der über einen Zeitraum von 24 bis
48 Stunden anhielt und anschließend auf einem Niveau von ca. 150
% des Ausgangswertes, unabhängig von der verwendeten Testmetho-
de, über einen Zeitraum bis zu 96 Stunden nach Versuchsbeginn
konstant blieb. Da bekannt ist, daß von Willebrand-Faktor gegen
den proteolytischen Abbau durch Plasmin empfindlich ist, mußte
angenommen werden, daß die Gabe von rtPA eine vermehrte von
Willebrand-Faktor-Inaktivierung, verbunden mit einer sekundären
Reduktion des Plasma-Faktor VIII-Spiegels durch Fehlen des Sta-
bilisierungsproteins für Faktor VIII zu finden sein wird. Die
gleichzeitige Gabe von Aprotinin als Inhibitor der Fibrinolyse
und des Plasmins ließ lediglich eine Inhibition des rtPA-Effek-
tes erwarten. Der scheinbar paradoxe Anstieg des Plasma-Faktor
VIII-Levels kann daher durch die Interferenz von Aprotinin
und/oder rtPA mit den Metabolisierungsmechanismen für Faktor
VIII erklärt werden.

B e i s p i e l   2 :

Ein von von Willebrand-Faktor-defizienter Hund wie in Beispiel 1
wurde narkotisiert und anschließend mit Aprotinin (Pantinol) mit
einer Dosis von 100.000 K.I.E. als intravenöser Bolus vorbehan-
delt. Anschließend wurde dem Tier ein rekombinantes von Wille-
brand-Faktor-Präparat in einer Dosis von 70 RcoF E/kg gegeben.
Die Herstellung und Charakterisierung des rekombinanten von
Willebrand-Faktor-Präparates ist in Fischer et al., FEBS Lett.
375:259 (1995), beschrieben. Unmittelbar nach der Gabe des re-
kombinanten von Willebrand-Faktor-Präparates wurde über drei
Stunden eine Infusion von 100.000 K.I.E. Aprotinin intravenös
verabreicht. Blutproben auf Zitrat wurden vor Beginn des Versu-
ches (= Zeitpunkt 0) 15 min, 30 min, 1 h, 3 h, 24 h und 48 h

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nach der Behandlung mit rekombinantem von Willebrand-Faktor ent-
nommen und daraus Plasma, wie in Beispiel 1 beschrieben, herge-
stellt und bis zur weiteren Analyse bei -20°C tiefgefroren. Von
diesen Blutproben wurde anschließend von Willebrand-Faktor-Anti-
gen unter Verwendung eines Kaninchen Anti-Human-von Willebrand-
Faktor-Antikörpers mit dem Test Asserachrom vWF, Boehringer
Mannheim, gemessen. Außerdem wurde die Ristocetin-Cofaktor-Akti-
vität, gemessen durch Ristocetin induzierte Aggregation Formal-
dehyd-fixierter humaner Blutplättchen und nach der Methode von
Evans und Austen, Brit. J. Hetamol. 37:289 (1977) bestimmt. Als
von Willebrand-Faktor-Standard diente ein humaner Plasma-Stan-
dard. Außerdem wurde Faktor VIII, wie in Beispiel 1 beschrieben,
getestet. Die Resultate der Bestimmung der Blutproben an von
Willebrand-Faktor und Faktor VIII-Aktivität sind der Fig. 2 zu
entnehmen. In der Grafik ist ein Mittelwert der Faktor VIII-Ak-
tivität, gemessen nach den beiden Faktor VIII-Bestimmungsmetho-
den, als Graph eingezeichnet. Von Willebrand-Faktor wurde, wie
aus der Literatur bekannt (Turecek et al., Blood 90: 3555
(1997)), mit einer Halbwertszeits von 10 bis 20 h eliminiert, so
daß nach 48 h kaum mehr von Willebrand-Faktor in der Zirkulation
nachweisbar war. Gleichzeitig mit Gabe des rekombinanten von
Willebrand-Faktors und des Aprotinins stieg der Plasma-Faktor
VIII-Spiegel innerhalb von 3 h auf ca. 150 % des Ausgangswertes
an und blieb dann auf diesem Niveau stabil oder zeigte sogar ei-
nen leicht steigenden Trend bis zu 48 h nach der Verabreichung.
Auch dieses Ergebnis kann wie Beispiel 1 interpretiert werden,
daß durch die Gabe des Aprotinins trotz fehlender Stabilisierung
des Faktor VIII durch den rekombinanten von Willebrand-Faktor,
durch eine Interferenz mit dem Faktor VIII-Metabolismus der Fak-
tor VIII-Plasma-Spiegel anhaltend erhöht bleibt.

B e i s p i e l   3 :

**Wirkung von RAP auf Faktor VIII-Gewinnung in Knock-out-Mäusen
mit schwerem Faktor VIII-Mangel**

Auf gentechnischem Wege wurde ein Mäusestamm mit schwerem Faktor
VIII (FVIII)-Mangel durch gezielte Disruption des Mäuse-Faktor
VIII-Gens gemäß Bi et al. Nature Genetics 10:119-121 (1995) ge-
schaffen. Faktor VIII-Knock-out-Mäuse wurden durch eine Inser-

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tion eines neo-Gens in das 3'-Ende von Exon 17 des Mäuse-Faktor
VIII-Gens geschaffen. Die betroffenen männlichen Tiere (X'Y)
hatten nicht nachweisbare Faktor VIII-Levels von <0,02 ± 0,01
E/ml, wenn Messungen entweder mit einem chromogenen Faktor VIII-
Test, Hyland Immuno, Wien, Österreich, wie kürzlich beschrieben
(Turecek et al., Thromb. Haemostas. Suppl. 769 (1997)) oder mit-
tels Antigen-ELISA, wie nachstehend beschrieben, vorgenommen
wurden.

Zwei betroffene, hemizygote männliche Mäuse (X'Y) wurden intra-
venös mit einer Dosis von 200 E/kg Körpergewicht einer rekombi-
nanten Human-Faktor VIII (rhFVIII)-Präparation behandelt, die
von Chinese Hamster-Ovarien-Zellen stammte, die wie beschrieben
hergestellt worden waren (PCT WO/85/01961), und ohne stabilisie-
rendes Protein pharmazeutisch formuliert worden war.

Unter Narkose wurden eine Stunde nach der Behandlung die
Schwanzspitzen der Mäuse mit der Klinge eines Skalpells, wie von
Novak et al., Brit. J. Haematol. 69:371-378 (1998) beschrieben,
eingeschnitten. Ein Volumen von 50 μl Blut wurde mit Kapillar-
röhrchen (Ringcaps, Hirschmann, Deutschland) aus den Schwanzwun-
den gesammelt, wobei die Kapillarröhrchen mit Lithium-Heparin
als Antikoagulans beschichtet waren. Die Kapillarröhrchen wurden
geschlossen und zentrifugiert, um die Blutzellen vom Plasma zu
trennen. Danach wurden die Kapillarröhrchen geöffnet, und die
Zell- und die Plasma-Fraktion wurden durch weiteres Zentrifugie-
ren gesammelt. Schließlich wurden die Plasmaproben der Faktor
VIII-Bestimmung mittels Faktor VIII-Antigen ELISA, Test-Set
IMMUNOZYM FVIII Ag, Hyland Immuno, Wien, Österreich, unter Ver-
wendung von monoklonalen Antifaktor VIII-Antikörpern sowohl für
das Abfangen wie für den Nachweis, wie beschrieben (Stel et al.,
Nature 303:530-532 (1983); Lenting et al., J. Biol. Chem. 269:
7150-7155 (1994); Leyte et al., Biochem. J. 263:187-194 (1989))
unterzogen. Die resultierenden Faktor VIII-Werte wurden in in-
ternationalen Einheiten von humanem Faktor VIII ausgedrückt. Die
Ergebnisse der Faktor VIII-Plasma-Level sind in der Tabelle an-
gegeben.

Zwei andere betroffene hemizygote männliche Mäuse (X'Y) wurden

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mit rekombinantem Rezeptor-assoziiertem Protein (GST-RAP) 10 min
vor der Behandlung mit dem rekombinanten humanen Faktor VIII mit
einer Dosis von 40 mg/kg Körpergewicht vorbehandelt. Das bei
dieser Untersuchung verwendete Rezeptor-verwandte Protein (RAP),
welches mit dem Low-Density-Lipoprotein-Rezeptor in Wechselwir-
kung tritt, wurde durch bakterielle Fermentation, wie von Hertz
et al., J. Biol. Chem. 266:21232-21238 (1991) beschrieben, er-
halten. Ein Fusionsprotein des RAP mit Glutathion-S-Transferase
wurde in E. coli exprimiert und mittels Affinitätschromatogra-
phie auf Glutathion-Agarose gereinigt. Das resultierende Protein
bestand hauptsächlich aus dem Fusionsprotein und Spaltprodukten
von RAP und Glutathion-S-Transferase. Das Fusionsprotein wurde
in einem injizierbaren Puffer bereit für eine Verabreichung an
die Faktor VIII-Knock-out-Mäuse formuliert. Wie bei der Kon-
trollgruppe (Behandlung nur mit Faktor VIII) wurden Blutproben
eine Stunde nach der Verabreichung von rekombinantem Faktor VIII
genommen und unter Verwendung von Faktor VIII-Antigen-ELISA auf
Faktor VIII-Aktivität gemessen. Die Ergebnisse sind in der Ta-
belle angeführt.

	Behandlung	Dosis	Gewinnung 1 h nach Behandlung
Maus Nr.	GST-RAP	rhFVIII	FVIII:Ag (E/ml Plasma)
1	40 mg/kg	200 E/kg	1,92
2	40 mg/kg	200 E/kg	1,88
3	-	200 E/kg	0,73
4	-	200 E/kg	0,83

Bei mit GST-RAP vorbehandelten Mäusen betrug die Faktor VIII-Ge-
winnung mehr als 200 % der Plasma-Level nach der Behandlung mit
rekombinantem Faktor VIII allein.

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P a t e n t a n s p r ü c h e :

1.   Pharmazeutische Präparation zur Behandlung von Blutgerin-
nungsstörungen, enthaltend mindestens ein Protein, ausgesucht
aus der Gruppe von einem pro-Protein der Blutgerinnung und wei-
ters einen gerinnungsphysiologisch inerten Rezeptor-Bindungskom-
petitor.

2.   Präparation nach Anspruch 1, dadurch gekennzeichnet, daß
das pro-Protein der Blutgerinnung ausgesucht ist aus der Gruppe
von Faktor II, V, VII, VIII, IX, X, XI, XII, vWF und Protein C.

3.   Präparation nach Anspruch 1, dadurch gekennzeichnet, daß
das Protein vWF ist.

4.   Präparation nach einem der Ansprüche 1 bis 3, dadurch ge-
kennzeichnet, daß das Protein aus einem biologischen Material
ausgesucht aus der Gruppe von Humanplasma, einer Plasmafraktion
und einem Zellkulturüberstand gewonnen ist.

5.   Präparation nach einem der Ansprüche 1 bis 4, dadurch ge-
kennzeichnet, daß der Rezeptor-Bindungskompetitor ein Ligand des
Lipoprotein-Rezeptor-Related-Proteins (LRP) ist.

6.   Präparation nach einem der Ansprüche 1 bis 5, dadurch ge-
kennzeichnet, daß der Rezeptor-Bindungskompetitor RAP ist.

7.   Präparation nach einem der Ansprüche 1 bis 6, dadurch ge-
kennzeichnet, daß der Rezeptor-Bindungskompetitor ein Gemisch
aus einem gerinnungsphysiologisch aktivem Protein und dessen In-
hibitor ist, insbesondere tPA und Aprotinin.

8.   Präparation nach einem der Ansprüche 1 bis 7, dadurch ge-
kennzeichnet, daß das Gemisch tPA und Aprotinin enthält.

9.   Präparation nach einem der Ansprüche 1 bis 8, dadurch ge-
kennzeichnet, daß es als Set zur Verfügung gestellt wird, ent-
haltend
        a) das Protein und

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    b) den Rezeptor-Bindungskompetitor.

10.   Präparation nach einem der Ansprüche 1 bis 9, dadurch gekennzeichnet, daß das Protein Faktor VIII ist und der Rezeptor-Bindungskompetitor Aprotinin, gegebenenfalls gemeinsam mit tPA.

11.   Präparation nach einem der Ansprüche 1 bis 9, dadurch gekennzeichnet, daß das Protein vWF ist und der Rezeptor-Bindungskompetitor Aprotinin ist.

12.   Präparation nach einem der Ansprüche 1 bis 11, dadurch gekennzeichnet, daß der Rezeptor-Bindungskompetitor ein humanphysiologisches extrazelluläres Protein ist, oder dessen Analoges.

13.   Präparation nach einem der Ansprüche 1 bis 12, dadurch gekennzeichnet, daß der Rezeptor-Bindungskompetitor ausgewählt aus der Gruppe der Enzyme, insbesondere Tissue-type Plasminogenaktivator (tPA), Urokinase (u-PA), pro-Urokinase (pro-u-PA), Lipoproteinlipase (LPL) und Kallikrein; Inhibitoren, insbesondere Plasminogen-Aktivator-Inhibitor-1 (PAI-1), Tissue factor pathway Inhibitor (TFPI) und Aprotinin; Enzym-Inhibitor-Komplexe, insbesondere t-PA-PAI-1, u-PA-PAI-1 und Thrombin-PAI-1, α_2-Macroglobulin-Proteinase (fast form) α_2M, α_2M·, Pregnancy zone protein-Proteinase, Elastase-α^1-Antitrypsin, Thrombin-Antithrombin III, Thrombin-Heparin-Cofactor II und u-PA-Protease-Nexin I; Lipoproteine, insbesondere low density-Lipoprotein (LDL), Apolipoprotein E angereichertes β-very low density-Lipoprotein, (apo E-β-VLDL), LPL angereichertes VLDL (LPL-VLDL) und LPL angereichertes β-VLDL (LPL-β-VLDL); Matrix-Proteine, insbesondere Thrombospondin 1 und 2; Toxine und Viren, insbesondere Pseudomonas Exotoxin A, und Minor-group human Rhinovirus; oder andere Liganden, insbesondere Apolipoprotein E (apo E), Lactoferrin, Rezeptor assoziiertes Protein (RAP), FVIII und vWF, ist.

14.   Kombinationspräparat enthaltend Aprotinin und tPA zur medizinischen Verwendung.

15. Verwendung eines Präparates nach einem der Ansprüche 1 bis 13 zur Herstellung eines Mittels zur Behandlung eines Patienten

- 16 -

mit einem phänotypischen Gerinnungsfaktor-Mangel.

16. Verwendung nach Anspruch 15, dadurch gekennzeichnet, daß der Patient vWF-defizient ist.

17. Verwendung eines Präparates nach einem der Ansprüche 1 bis 13 zur Herstellung eines Mittels zur Verlängerung der biologischen Halbwertszeit des Proteins in vivo.

18. Verwendung eines Präparates nach Anspruch 17 enthaltend Aprotinin und tPA.

19. Verwendung nach Anspruch 17, dadurch gekennzeichnet, daß das Protein Blutgerinnungsfaktor VIII ist.

20. Verwendung einer pharmazeutischen Präparation eines LRP-Liganden zur Herstellung eines Mittels zur Behandlung eines Patienten mit phänotypischem Gerinnungsfaktor-Mangel.

21. Verwendung eines Präparates nach Anspruch 20 enthaltend Aprotinin und tPA.

22. Verwendung nach Anspruch 20, dadurch gekennzeichnet, daß der Patient vWF-defizient ist.

23. Verwendung einer pharmazeutischen Präparation eines LRP-Liganden zur Herstellung eines Mittels zur Verlängerung der biologischen Halbwertszeit eines Proteins.

24. Verwendung einer pharmazeutischen Präparation nach Anspruch 23 enthaltend Aprotinin und tPA.

25. Verwendung nach Anspruch 23, dadurch gekennzeichnet, daß das Protein Blutgerinnungsfaktor VIII ist.

Wirkung von Aprotinin und rtPA bei einem vWF-defizienten Hund

FIG.1

Wirkung von Aprotinin und rvWF bei einem vWF-defizienten Hund

FIG.2

(54) Title: METHODS AND COMPOSITIONS FOR INHIBITION OF HEPATIC CLEARANCE OF TISSUE-TYPE PLASMINOGEN ACTIVATOR

(57) Abstract

Methods and compositions for inhibiting the hepatic clearance of tissue-type plasminogen activator (t-PA) _in vivo_ by administering a t-PA-hepatic clearance-inhibiting amount of 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment thereof, or genetically or chemically modified forms of the 39kDa protein or fragments thereof are described. Compositions for treatment of thrombolytic diseases comprised of t-PA and a t-PA-hepatic clearance-inhibiting effective amount of 39kDa protein, a t-PA-hepatic clearance inhibiting fragment thereof, and genetically or chemically modified forms of the 39kDa protein or its fragments are described.

TITLE

METHODS AND COMPOSITIONS FOR INHIBITION OF
HEPATIC CLEARANCE OF TISSUE-TYPE PLASMINOGEN ACTIVATOR

TECHNICAL BACKGROUND

This invention was made with U.S. Government support under HL 17646 awarded by the National Institutes of Health. The U.S. Government has certain rights in the invention.

FIELD OF THE INVENTION

5

This invention relates to methods and compositions for inhibiting the hepatic clearance of tissue-type plasminogen activator (t-PA) *in vivo* comprising administering a t-PA-hepatic clearance-inhibiting amount of 39kDa protein or a t-PA-hepatic

10   clearance-inhibiting fragment thereof, or genetically or chemically modified forms of the 39kDa protein or fragments thereof; and to methods and compositions for treatment of thrombolytic diseases comprising administering t-PA and a t-PA-hepatic clearance-inhibiting effective amount of 39kDa protein,

15   a t-PA-hepatic clearance inhibiting fragment thereof, and genetically or chemically modified forms of the 39kDa protein or its fragments.

BACKGROUND OF THE INVENTION

Tissue plasminogen activator is an enzyme widely used

20   as a thrombolytic agent in the treatment of acute myocardial infarction. t-PA is secreted from endothelial cells as a single polypeptide chain that is subsequently cleaved (between Arg_{275} and Ile_{276}) into two chains held together by a single disulfide bond (Rijken, D.C. et al., (1981) J. Biol. Chem. 256, 7035-7041.

25   Both the single- and two-chain forms of the enzyme can bind to fibrin (Rijken, D.C. et al., (1982) J. Biol. Chem. 257, 2920-

2925; Higgins, D.L. et al., (1987) Biochem. 26, 7786-7791),

although the two-chain form is catalytically more active

(Wallen, P. et al., (1982) Biochim. Biophys. Acta 719, 318-328;

Ranby, M., (1982) Biochim Biophys. Acta 704, 461-469; Tate, K.M.

5   et al., (1987) Biochem. 26, 338-343; and Petersen, L.C. et al.,

(1988) Biochim. Biophys. Acta 952, 245-254). In addition, both

forms of the enzyme (Jorgensen, M. et al., (1987) Thromb.

Haemostasis 58, 872-878) are inactivated by a fast-acting

plasminogen activator inhibitor (type 1)(PAI-1), a member of the

10   serpin family that is secreted from endothelial cells and forms

a covalent bond with Ser_{478} of t-PA (Levin, E.G., (1983) Proc.

Natl. Acad. Sci. USA 80, 6804-6808; and Sprengers, E.D. et al.,

(1987) Blood 69, 381-387).

        Exogenously administered t-PA is capable of eliciting

15   prompt thrombolysis in therapeutic doses that do not produce

marked fibrinolysis in experimental animals with induced

coronary artery thrombosis (Bergmann, S.R. et al., (1983)

Science, 220, 1181-1183) and in patients with evolving

myocardial infarction (Van de Werf, F. et al., (1984) New Eng.

20   J. Med. 310, 609-613; Collen, D. et al., (1984) Circ. 70, 1012-

1017; Van de Werf, F. et al., (1984) Circ. 69, 605-610;

Thrombolysis in Myocardial Infarction Study Group, (1985) New

Eng. J. Med. 312, 932-936; and European Cooperative Study Group,

(1985) Lancet 1, 842-847). However, because the clearance of

25   t-PA from the circulation is so rapid, continuous infusions have

been required.

        In vivo studies of t-PA clearance have been performed

in a variety of species including mice (Fuchs, H.E. et al.,

(1985) Blood 65, 539-544); rats (Emeis, J.J. et al., (1985)
Thromb. Haemost. 54, 661-664; Rijken, D.C. et al., (1986)
Biochem. J. 238, 643-646; Kuiper, J. et al., (1988) J. Biol.
Chem. 263, 18220-18224; Bakhit, C. et al., (1988) Fibrin. 2, 31-
5   36; Krause, J. et al., (1990) Biochem. J. 267, 647-652); rabbits
(Korninger, C. et al., (1981) Thromb. Haemostasis 46, 658-661;
Bounameaux, H. et al., (1986) Blood 67, 1493-1497); dogs
(Devries, S.R. et al., (1987) Fibrin. 1, 17-21; Yasuda, T. et
al., (1988) J. Clin. Invest. 81, 1284-1291); and monkeys
10   (Flameng, W. et al., (1985) J. Clin. Invest. 75, 84-90). These
studies together with experiments in man (Garabedian, H.D. et
al., (1986) Am. J. Cardiol. 58, 673-679; Verstraete, M. et al.,
(1986) Thromb. Haemostas. 56, 1-15) demonstrate the rapid
removal of t-PA from the circulation, which varies from about $t_{1/2}$
15   = 1 min in rats to $t_{1/2}$ = 5 min in man.

The liver appears to be the major site of removal and
catabolism of t-PA (Nilsson, T. et al., (1984) Scand. J.
Haematol. 33, 49-53; Devries, S.R. et al., (1987) Fibrin. 1, 17-
21; Korninger, C. et al., (1981) Thromb. Haemostasis 46, 658-
20   661; Bounameaux, H. et al., (1986) Blood 67, 1493-1497; Beebe,
D.P. et al., (1986) Thromb. Res. 43, 663-674; Nilsson, S. et
al., (1985) Thromb. Res. 39, 511-521; Emeis, J.J. et al., (1985)
Thromb. Haemost. 54, 661-664; Rijken, D.C. et al., (1986)
Biochem. J. 238, 643-646; Fuchs, H.E. et al., (1985) Blood 65,
25   539-544; and Kuiper, J. et al., (1988) J. Biol. Chem. 263,
18220-18224). About 80% of exogenous t-PA delivered
intravascularly rapidly accumulates in the liver and is
subsequently degraded, with subsequent appearance of degradation

products in plasma. These studies support a general clearance
mechanism for t-PA in which uptake and degradation within the
liver is followed by the release of the degradation products
initially into the blood and subsequently into the urine.

5   Further, the half-life of circulating t-PA is markedly
prolonged in animals subjected to hepatectomy (Bounameaux, H. et
al., (1986) Blood 67, 1493-1497; and Nilsson, T. et al., (1984)
Scand. J. Haematol. 33, 49-53). Neither the protease active
site nor a specific glycosylation pattern appears to be a major
10   determinant of hepatic recognition and degradation of t-PA *in
vivo* (Fuchs, H.E. et al., (1985) Blood 65, 539-544), in perfused
liver systems (Emeis, J.J. et al., (1985) Thromb. Haemost. 54,
661-664, or in isolated hepatocytes (Bakhit, C. et al., (1987)
J. Biol. Chem. 262, 8716-8720. The clearance and catabolism of
15   t-PA has been reviewed in detail (Krause, J. (1988) Fibrin. 2,
133-142). However, information is limited regarding the
particular cell type responsible for clearance of t-PA.

               After administration of fluorescent or radiolabelled
t-PA to rats and subfractionation of the livers into
20   parenchymal, endothelial, and Kupffer cells, it was found that
parenchymal and endothelial cells constitute the major sites for
hepatic uptake (Fuchs, H.E. et al., (1985) Blood 65, 539-544;
Sprengers, E.D. et al., (1987) Blood 69, 381-387; Kuiper, J. et
al., Fibrin, 2:28 (1988) and Bugelski, P.J. et al., (1989)
25   Thromb. Res. 53, 287-303).

               The uptake of t-PA into all liver cell types is
inhibited by *in vivo* competition with unlabelled t-PA whereas
glycoproteins such as mannan and ovalbumin inhibit the specific

uptake of labelled t-PA in isolated liver endothelial cells.

The endocytosis of t-PA is mediated, at least in part, by

mannose receptors on endothelial cells (Einarsson, M. et al.,

(1985) Thromb. Haemost. 54, 270; and Kuiper, J. et al., (1988)

5   Fibrin. 2, 28). Monensin, NH_4Cl, and cytochalasin B block the

uptake and degradation of t-PA, indicating that the uptake is

endocytotic and that the degradation is lysosomal. In hepatoma

cell lines, representing parenchymal cells, t-PA clearance

involves ligand binding, uptake, and degradation mediated by a

10   high capacity, high-affinity specific receptor system (Owensby,

D.A. et al., (1988) J. Biol. Chem. 263, 10587-10594).

Subfractionation of rat liver parenchymal, endothelial, and

Kupffer cells 5 minutes after ^{125}I-t-PA injection revealed that

liver parenchymal cells are responsible for about 55% of the

15   cleared ^{125}I-t-PA, endothelial cells for about 40%, and Kupffer

cells for about 5% (Kuiper, J. et al., (1988) J. Biol. Chem.

263, 18220-18224; Rijken, D.C. et al., (1990) Thromb. Res.

Suppl. X, 63-71).

       Two distinct mechanisms for t-PA catabolism by

20   hepatoma cells have been shown. t-PA complexed to PAI-1 is

recognized by a PAI-1 dependent receptor on the cell surface of

human hepatoma HepG2 cells (Schwartz, A.L. et al., (1981) J.

Biol. Chem. 256, 8878-8881; Owensby, D.A. et al., (1988) J.

Biol. Chem. 263, 10587-10594; Morton, P.A. et al., (1989) J.

25   Biol. Chem. 264, 7228-7235; Bu, G. et al., (1992) J. Biol. Chem.

267, 15595-15602). t-PA in the absence of bioactive PAI-1 has

been found to bind to PAI-1 independent receptors which mediate

binding and endocytosis of t-PA on rat hepatoma MH_1C_1 cells (Bu,

6

G. et al., (1992) J. Biol. Chem. 267, 15595-15602) and on rat

Novikoff hepatoma cells (Nguyen, G. et al., (1992) J. Biol.

Chem. 267, 6249-6256). Although this PAI-1 independent t-PA

clearance system has not been reported on human hepatocytes, the

5   rapid clearance of intravenously injected t-PA, normally at a

level far exceeding the available PAI-1, suggests the existence

of a PAI-1 independent t-PA clearance system.

At present, t-PA is administered clinically in the

form of an initial bolus that is followed by sustained infusion.

10   The total amount of enzyme administered during a standard 3 hour

treatment is 50-100 mg. Such large amounts are required for two

reasons: first, to counterbalance the effects of the rapid

clearance of t-PA from the circulation, and second, to overcome

the effects of high concentrations of fast-acting inhibitors of

15   the enzyme that are present in plasma and platelets. When high

doses are used in an effort to increase the rate of clot lysis

or to lyse refractory clots, there is a risk of systemic

fibrinolysis which affects the body's capacity to stop bleeding

and hemorrhage.

20   Reducing the rate of t-PA clearance from the

circulation following administration would provide a significant

advantage in clinical use. This would allow t-PA to be

administered in much smaller doses than are currently required,

thereby reducing, e.g., the risk of systemic fibrinolysis and

25   hemorrhage. Accordingly, an objective of the invention is to

provide methods and compositions for the inhibition of hepatic

clearance of t-PA. Another objective of the invention is to

provide a method and composition for the treatment of

thrombolytic diseases which allows t-PA to be administered in much smaller doses than currently required. These and other objectives and f atures of the invention will be apparent from the following description.

5   BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a graph of the saturation binding of ^{125}I-39kDa protein to MH_1C_1 cells in the presence or absence of 0.5μM unlabeLled 39kDa protein. (*circles*) = total ligand binding; (*triangles*) = non-specific ligand binding. Specific

10   binding (*squares*) was calculated as the difference between total and non-specific binding. Each symbol represents the mean of triplicate determinations. The *inset* is a Scatchard plot of specific binding where B = bound ^{125}I-39kDa protein and B/F = bound/free ^{125}I-39kDa protein. FIG. 1B is a SDS-PAGE gel (10%

15   acrylamide, non-reduced) of equivalent volumes of post-binding cell lysates, each from about 60,000 cells. The position of ^{125}I-39kDa protein is indicated by a closed arrow.

FIGS. 2A-B are graphs of the saturation binding of ^{125}I-t-PA and ^{125}I-39kDa protein to HepG2 cells. FIG. 2A is a

20   graph of the saturation binding of ^{125}I-t-PA in the absence or presence of 1 μM unlabelled t-PA. (*squares*) = total ligand binding; (*triangles*) = non-specific ligand binding; (*circles*) = specific binding calculated as the difference between total and non-specific binding. *Symbols* represent the means of triplicate

25   determinations. The *inset* is a Scatchard plot of specific binding, where B = bound ^{125}I-t-PA, B/F = bound/free ^{125}I-t-PA. FIG. 2B is a graph of the saturation binding of the ^{125}I-39kDa

protein.   The same symbols are used as in FIG. 2A.   The *inset*,
like FIG. 2A, is a Scatchard plot of specific binding.

          FIGS. 3A-D are SDS-PAGE gels (8.5% acrylamide) of
specific cross-linking of ^{125}I-39kDa protein to low density
5   lipoprotein receptor-related protein (LRP) in the presence and
absence of 0.5μM unlabelled 39kDa protein.   In FIGS. 3A and 3B,
MH_1C_1 cells were incubated in the presence of 0.5mM DTSSP for 1
hour.   In FIGS. 3C and 3D, MH_1C_1 cells were incubated in the
absence of 0.5mM DTSSP for 1 hour.   Cells were then lysed and
10   immunoprecipitated with normal rabbit serum (*lanes 1 and 4*),
anti-LRP serum (*lanes 2 and 5*), or anti-39kDa protein serum
(*lanes 3 and 6*).   In FIGS. 3A and 3C, the SDS-PAGE gels were
analyzed under nonreducing conditions.   In FIGS. 3B and 3D, the
SDS-PAGE gels were analyzed under reducing conditions.   Gels
15   from samples incubated in the presence of cross-linker were
autoradiographed for 3 days at -70°C; gels incubated in the
absence of cross-linker were autoradiographed for only 2 days.
The position of ^{125}I-39kDa protein (*closed arrowhead*) is
indicated.

20   FIGS. 4A-B are SDS-PAGE gels of chemical cross-linking
of ^{125}I-t-PA:PAI-1 to LRP on HepG2 cells.   (N.R.) = normal rabbit
serum; (α-t-PA) = anti-t-PA antibody; (α-PAI-1) = anti-PAI-1
antibody; (α-LRP) = anti-LRP antibody.   FIG. 4A is a SDS-PAGE
gel (7.5% acrylamide) analyzed under nonreducing conditions.
25   FIG. 4B is a SDS-PAGE gel (7.5% acrylamide) analyzed under
reducing conditions.   The region of cross-linked material is
marked with a bracket. The positions of ^{125}I-t-PA (*closed arrow*)
and ^{125}I-t-PA:PAI-1 (*open arrow*) are indicated.

FIG. 5A is a graph of the inhibition of 3 nM ^{125}I-t-PA binding to H pG2 cells by the 39kDa protein in the absence or presence of increasing concentrations of competitor proteins. Each symbol represents the average of duplicate determinations

5   and the standard deviations are less than 5%. FIG. 5B is a SDS polyacrylamide gel (7.5%) of post-binding buffers overlying cell monolayers and corresponding cell lysates where the gel was analyzed under nonreducing conditions using a sample buffer containing low concentration of SDS (0.2%). Samples were

10   selected from either no competitor protein (lanes 1 and 5), or with 500 nM t-PA (lanes 2 and 6), 500 nM 39kDa protein (lanes 3 and 7), or 500 nM BSA (lanes 4 and 8).

FIG. 6 are SDS-PAGE gels of chemical cross-linking of t-PA:PAI-1 to LRP on ^{35}S-methionine-labelled HepG2 cells which

15   were analyzed under nonreducing or reducing conditions. (N.R.) = normal rabbit serum (*lanes 1, 6, 11*); (α-t-PA) = anti-t-PA antibody (*lanes 2, 7, 12*); (α-PAI-1) = anti-PAI-1 antibody (*lanes 3, 8, 13*); (α-39kDa) = anti-39kDa protein antibody (*lanes 4, 9, 14*); (α-LRP) = anti-LRP antibody (*lanes 5, 10, 15*). The

20   regions of cross-linked material in the non-reducing gel are marked with brackets. The following positions are indicated: LRP large subunit (515 kDa) (*2 closed arrowheads*); LRP small subunit (85 kDa) (*1 closed arrowhead*); PAI-1 (*1 closed arrow*); and t-PA:PAI-1 complex (*1 open arrow*).

25   FIGS. 7A-B are SDS-PAGE gels (8.5% acrylamide) of cross-linking of unlabelled 39kDa protein to the cell surface of metabolically labelled rat MH$_1$C$_1$ cells. Cell monolayers in lanes 4-6 w re incubated for 1 hour with 0.5 mM DTSSP, whereas cell

monolayers in lanes 1-3 were incubated for 1 hour in PBSc alone.
Cells were then lysed and immunoprecipitated with either normal
rabbit serum (lanes 1 and 4), affinity purified α-rat 39kDa
protein (lanes 2 and 5), or affinity purified α-human LRP (lanes

5   3 and 6). In FIG. 7A, the SDS-PAGE gel was analyzed under
nonreducing conditions. In FIG. 7B, the SDS-PAGE gel was
analyzed under reducing conditions. The closed arrowhead
indicates the position of the 39kDa protein. The open and
closed arrows indicate, respectively, the positions of the 85kDa

10   and 520kDa subunits of LRP.

       FIGS. 8A-F are SDS-PAGE gels (8.5% acrylamide) of
co-binding and cross-linking of ^{125}I-39kDa protein and unlabelled
t-PA to rat MH_1C_1 cells in the presence or absence of 0.5 μM
unlabelled 39kDa protein. In FIGS. 8A, 8C, and 8E, the SDS-PAGE

15   gels were analyzed under nonreducing conditions. In FIGS. 8B,
8D, and 8F, the SDS-PAGE gels were analyzed under reducing
conditions.

       FIG. 9 is a graph of rapid endocytosis and degradation
of surface bound ^{125}I-39kDa protein by MH_1C_1 cells in the presence

20   and absence of 0.5 μM unlabelled 39kDa protein. (circles,
degraded ligand) = TCA soluble radioactivity; (diamonds, both
dissociated and degraded ligand) = total radioactivity;
(squares, plasma membrane associated) = Pronase sensitive
ligand; (triangles, cell associated) = Pronase resistant ligand.

25   Each data point represents specific radioactivity (i.e., the
difference of total and non-specific radioactivity) and is the
mean of triplicate determinations.

FIGS. 10A-B are graphs of the inhibition of ^{125}I-t-PA

uptake and degradation by the 39kDa protein on HepG2 cells.

(*open squares*) = prewarmed (37°C) binding buffer containing

^{125}I-t-PA (3 nM); (*open circles*) = prewarmed (37°C) binding buffer

5   containing ^{125}I-t-PA (3 nM) in the presence of 1 μM concentration

of t-PA; (*open triangles*) = prewarmed (37°C) binding buffer

containing ^{125}I-t-PA (3 nM) in the presence of 1 μM concentration

of the 39-kDa protein; (*closed circles*) = prewarmed (37°C)

binding buffer containing ^{125}I-t-PA (3 nM) in the presence of 1 μM

10   concentration of BSA.   FIG. 10A shows TCA soluble radioactivity

representing the degraded ligands in fmoles equivalents of t-PA.

FIG. 10B shows the radioactivity associated with each cell

lysate in fmoles equivalents of t-PA.   In each graph, each

symbol represents the average of triplicate determinations and

15   the standard deviations were less than 5%.

FIGS. 11A-B are graphs of the inhibition of ^{125}I-t-PA·

binding by rat 39kDa protein on MH_1C_1 cells in the presence of

increasing concentrations of either unlabelled t-PA or 39kDa

protein.   Each point represents the mean of triplicate

20   determinations.   FIG. 13A shows the inhibition of ^{125}I-t-PA

binding by the 39kDa protein.   FIG. 13B shows the inhibition of

^{125}I-t-PA binding by unlabelled t-PA.

FIG. 12 is a graph of the rapid uptake and degradation

of ^{125}I-39kDa protein by rat MH_1C_1 cells at 37°C.   *Closed circles*

25   represent TCA soluble counts (internalized and degraded ligand)

in the extracellular media.   *Triangles* indicate the amount of

cell associated radioactivity as determined following lysis of

the cell monolayers.   *Open circles* represent the sum of both

cell associated and extracellular degraded ligand. Each point

is the mean of triplicate determinations.

FIG. 13 is a graph of the inhibition of the ^{125}I-39kDa

protein by t-PA. Each symbol represents the average of

5   duplicate determinations and the standard deviations are less

than 5%.

FIG. 14 is a graph of the inhibition of 3 nM ^{125}I-t-PA

binding to HepG2 cells by anti-LRP antibody. (*open circles*) =

rabbit non-immune IgG; (*closed circles*) = rabbit polyclonal

10   anti-LRP IgG. Each symbol represents the average of duplicate

determinations and the standard deviations are less than 5%.

FIG. 15A is a Coomassie-stained SDS-PAGE gel of

purified 39kDa protein (10 μg). FIG. 15B is a graph of the

inhibition of ^{125}I-t-PA binding to rat liver MH_1C_1 cells by 39kDa

15   protein.

FIGS. 16A-B are graphs of the plasma clearance of

^{125}I-t-PA in rat *in vivo* in the absence or presence of unlabelled

t-PA or 39kDa protein. Bars = S.E.M. FIG. 16A shows the plasma

clearance of ^{125}I-t-PA alone (n=8) (*open circles*); the plasma

20   clearance of ^{125}I-t-PA when 12 nmol unlabelled t-PA is

administered prior to ^{125}I-t-PA (n=3) (*closed circles*); and the

plasma clearance of ^{125}I-t-PA when 250 nmol unlabelled 39kDa

protein is administered prior to ^{125}I-t-PA (n=4) (*closed

triangles*). FIG. 16B shows the plasma clearance of control

25   proteins ^{125}I-asialorosomucoid (*closed diamonds*) and

^{125}I-orosomucoid (*open diamonds*).

FIG. 17 is a graph of the plasma clearance of ^{125}I-39kDa

protein in rat *in vivo* in the presence of unlabelled 39kDa

protein.   (O) = administration of ^{125}I-39kDa protein alone (n=6);
( ) = administration of 12.5 nmol unlabelled 39kDa protein prior
to ^{125}I-39kDa protein (n=4); (■) = administration of 50 nmol
unlabelled 39kDa protein prior to ^{125}I-39kDa protein (n=4); (▲) =

5   administration of 125 nmol unlabelled 39kDa protein prior to
^{125}I-39kDa protein (n=2).

FIG. 18 is a graph of the inhibition of ^{125}I-t-PA
binding to rat liver MH_1C_1 cells by intact 39kDa protein, 20kDa
N-terminal fragment and 28kDa C-terminal fragment.   (●) = intact

10   39kDa protein; (■) = the 20kDa N-terminal fragment; (▲) = the
28kDa C-terminal fragment; and (O) = PVDF-elution buffer alone.

SUMMARY OF THE INVENTION

The present invention provides a method of inhibiting
the hepatic clearance of t-PA *in vivo* in humans by the

15   administration of a t-PA-hepatic clearance-inhibiting amount of
a 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment
thereof.   The 39kDa protein and fragments thereof may be
genetically or chemically modified.

An advantage of this method is that the plasma half-

20   life of t-PA is significantly increased.

The present invention also provides a 28kDa protein
and a chemically synthesized gene encoding this protein.   It has
been found that this 28kDa protein inhibits hepatic clearance of
t-PA.

25   The present invention also provides a method of
thrombolysis in a mammal by the administration of a
thrombolytically effective amount of t-PA and a t-PA-hepatic

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clearance-inhibiting effective amount of 39kDa protein or a
t-PA-hepatic clearance-inhibiting fragment thereof.

An advantage of this method is that the inhibition of
hepatic clearance of t-PA enables smaller doses of t-PA to be
5   used to achieve the same level of thrombolysis.

The present invention further provides a
pharmaceutical composition for a mammalian patient comprising
t-PA and a t-PA- hepatic clearance-inhibiting amount of 39kDa
protein or a t-PA-hepatic clearance-inhibiting fragment thereof.

10   An advantage of this pharmaceutical composition is
that the inclusion of a t-PA-hepatic clearance-inhibiting
protein enables a smaller dosage amount of t-PA to be used with
both physiological and cost benefits for the patient.

The present invention also provides a composition for
15   treating thrombolytic diseases in a mammal comprising t-PA and a
t-PA-hepatic clearance-inhibiting amount of 39kDa protein or a
t-PA-hepatic clearance-inhibiting fragment thereof.

An advantage of this composition is that the amount of
t-PA effective for treating thrombolytic diseases is markedly
20   reduced in comparison to current requirements.

Upon further study of the specification and appended
claims, further objectives and advantages of this invention will
become apparent to those skilled in the art.

DETAILED DESCRIPTION OF THE INVENTION

25   It is to be understood that while the present
invention is primarily contemplated for humans, it is also
contemplated for use in veterinary medicine.

In accordance with the invention, a 39kDa protein which competitively binds the hepatic receptor for t-PA has been found. By binding to the hepatic receptor for t-PA, the 39kDa protein prevents t-PA from being bound to the receptor and removed from the circulating plasma by endocytosis. This increases the plasma half-life of t-PA thereby prolonging t-PA's therapeutic effectiveness. An increase in the plasma half life of t-PA means that a smaller amount of t-PA may be used, which reduces the risk of systemic fibrinolysis and hemorrhage. As t-PA is very expensive, a significant cost savings can be achieved which, in turn, increases the availability of t-PA for clinical use.

The 39kDa protein is an active, effective, competitive binding agent for the hepatic receptor for t-PA. This t-PA-hepatic clearance-inhibiting protein is characterized by binding to LRP and inhibiting the binding of t-PA to LRP up to about 80% (K_i of about 0.5 nM) (see Examples 5-8 and 10, *infra*). Fragments of this 39kDa protein, particularly a 28kDa protein fragment, also competitively bind to the t-PA hepatic receptor. When the 39kDa protein or t-PA-hepatic clearance inhibiting fragment thereof is employed in the present invention, the standard dose of t-PA is reduced up to 90%.

Hepatic clearance of t-PA is inhibited *in vivo* in humans by administering a t-PA-hepatic clearance-inhibiting amount of the 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment of the 39kDa protein. The mode of administration is preferably intravenous. The preferred amount of 39kDa protein or fragment administered to the human to

16

inhibit hepatic clearance is in the range of about 60 to 6,000

mg/kg of body weight/dose. When the fragment of the 39kDa

protein is the 28kDa protein, the preferred amount administered

to the human to inhibit hepatic clearance is in the range of

5   about 38 to 3,800 mg/kg of body weight/dose. The 39kDa protein

or its fragments may be administered to the human concurrently

with t-PA, but is preferably administered up to 20 minutes prior

to the administration of t-PA. Administration of the 39kDa

protein or its fragments reduces hepatic clearance of t-PA

10   between 20 and 100%. The reduction in hepatic clearance is

measured by the increase in plasma half-life of t-PA.

It is to be understood that modified forms of the

39kDa protein and fragments thereof which inhibit hepatic

clearance of t-PA that are made by chemically or genetically

15   modifying the amino acid sequence of the 39kDa protein or its

fragments are encompassed within the scope of the present

invention. Such modified forms are characterized by their

ability to bind to LRP and to reduce hepatic clearance of t-PA

between 20 and 100%.

20   The 39kDa protein of the present invention has the

following amino acid sequence set forth in the Sequence Listing

as SEQ ID NO: 1:

```
1                 10                 20                 30
Y S R E K N Q P K P S P K R E S G E E F R M E K L N Q L W E K A Q

25             40                 50                 60
R L H L P P V R L A E L H A D L K I Q E R D E L A W K K L K L D G

       70                 80                 90
L D E D G E K E A R L I R N L N V I L A K Y G L D G K K D A R Q V

100                110                120                130
30   T S N S L S G T Q E D G L D D P R L E K L W H K A K T S G K F S G
```

```
                140                 150                 160
E  E  L  D  K  L  W  R  E  F  L  H  H  K  E  K  V  H  E  Y  N  V  L  L  E  T  L  S  R  T  E  E  I

                170                 180                 190
H  E  N  V  I  S  P  S  D  L  S  D  I  K  G  S  V  L  H  S  R  H  T  E  L  K  E  K  L  R  S  I  N

        200                 210                 220                 230
Q  G  L  D  R  L  R  R  V  S  H  Q  G  Y  S  T  E  A  F  E  E  P  R  V  I  D  L  W  D  L  A  Q

                240                 250                 260
S  A  N  L  T  D  K  E  L  E  A  F  R  E  E  L  K  H  F  E  A  K  I  E  K  H  N  H  Y  Q  K  Q  L

                270                 280                 290
E  I  A  H  E  K  L  R  H  A  E  S  V  G  D  G  E  R  V  S  R  S  R  E  K  H  A  L  L  E  G  R  T

        300                 310                 320
K  E  L  G  Y  T  V  K  K  H  L  Q  D  L  S  G  R  I  S  R  A  R  H  N  E  L
```

The 39kDa protein was prepared as shown in Example 1.

EXAMPLE 1

Purification of the 39kDa Protein

The procedure for purification of the 39kDa protein
from strains of *E.coli* carrying the over-expression plasmid
pGEX-39kDa has been described in Herz, J. et al., (1991)
J. Biol. Chem. 266, 21232-21238. A modified version of that
procedure, described below, was employed.

Cultures of *E.coli* strain DH5α carrying the
over-expression plasmid *pGEX-39kDa* were grown to mid-log phase
in LB medium with 100 µg/ml ampicillin at 37°C. Cultures were
cooled to 30°C and supplemented with 0.01%
isopropylthio-β-D-galactoside to induce expression of the
glutathione-S-transferase-39kDa fusion protein. Following a 4-6
hour induction at 30°C, cultures were cooled on ice and
collected by centrifugation.

All of the following steps were carried out at 4°C.
Cell pellets were lys d in PBSa with 1% Triton X-100, 1 µM
pepstatin, 2.5 µg/ml leupeptin, 0.2 mM phenylmethylsulfonyl

fluoride (PMSF), and 1 μM ethylenediaminetetraacetate (EDTA).
Sonication of this lysate was performed using a Branson Model
450 Sonifier, with the resulting membranes and other cellular
debris collected by centrifugation at 15,000 g for 15 minutes.

5   The supernatant from this step was incubated overnight with
agarose immobilized glutathione beads (Sigma Chemical Co.) in
PBSa and 0.1% sodium azide. The beads were then washed, and
elution of the fusion protein was carried out by competition
with 5 mM reduced glutathione (Sigma Chemical Co.). Following

10   dialysis, the fusion protein was cleaved by an overnight
incubation with 100 ng of activated human thrombin per 50 μg of
fusion protein. The glutathione-S-transferase epitope was
subsequently removed by further incubation with agarose
immobilized glutathione beads.

15   *   *   *   *   *

        The 28kDa protein fragment of the 39kDa protein
("28kDa protein") of the present invention has the following
amino acid sequence set forth in the Sequence Listing as SEQ ID
NO: 2:

20   1   10   20   30
     P R L E K L W H K A K T S G K F S G E E L D K L W R E F L H H K

                  40   50   60
     E K V H E Y N V L L E T L S R T E E I H E N V I S P S D L S D I K

              70   80   90
25   G S V L H S R H T E L K E K L R S I N Q G L D R L R R V S H Q G Y

         100   110   120   130
     S T E A E F E E P R V I D L W D L A Q S A N L T D K E L E A F R

                  140   150   160
     E E L K H F E A K I E K H N H Y Q K Q L E I A H E K L R H A E S V

30   170   180   190
     G D G E R V S R S R E K H A L L E G R T K E L G Y T V K K H L Q D

```
    200              209
L S G R I S R A R H N E L
```

The 28kDa protein is characterized by a molecular

weight of 28,000 daltons on SDS-PAGE, stability to acid

5   hydrolysis, solubility in 1% Triton X-100, and having

approximately the same inhibitory activity (K_i) on t-PA binding

to the hepatic receptor as the 39kDa protein. The 28kDa protein

may be cloned and purified as shown in the following example.

<div align="center">

EXAMPLE 2

Cloning of the 28kDa Protein

</div>

10   The 28kDa protein is produced with a bacterial

expression system. The gene encoding this protein is

synthesized using polymerase chain reaction (PCR) with the

following primers set forth in the Sequence Listing as SEQ ID

15   NO: 3 and SEQ ID NO: 4, respectively:

5'CCGCGTGGATCCCCCAGGCTGGAAAAGCTGTGG3',

5'TCAATGAATTCTCAGAGTCGCTCGCCGTCGCCCAC3'.

These PCR primers contain built-in restriction sites (BamH1 and

EcoR1, respectively). The PCR product after restriction enzyme

20   digestion is cloned directly to the pGEX-2T vector (Pharmacia).

Other bacterial expression vectors may be used. The constructed

plasmid is used to transform bacteria *E. coli* strain DH5αF' and

this bacterial transformant bearing the recombinant plasmid is

used to produce the 28kDa protein using the procedure of Example

25   1.

<div align="center">

*   *   *   *   *

</div>

Using standard recombinant techniques, a chemically

synthesized gene encoding the 28kDa protein may be prepared.

The chemically synthesized gene comprises a chemically

synthesized polynucleotide which codes on expression for the

amino acid sequence of the 28kDa protein given above.

A rat protein has also been found which binds to LRP

and inhibits binding of t-PA to the LRP hepatic receptor. This

5 rat protein has the following amino acid sequence set forth in

the Sequence Listing as SEQ ID NO: 5:

```
1                   10                  20                  30
P R L E K L W H K A K T S G S V R L T S C A R V L H K E K I H E Y

                    40                  50                  60
N V L L D T L S R A E E G Y E N L L S P S D M T H I K S D T L A S

                    70                  80                  90
K H S E L K D R L R S I N Q G L D R L R K V S H Q L R P A T E F E

100                 110                 120                 130
E P R V I D L W D L A Q S A N F T E K E L E S F R E E L K H F E A

                    140                 150                 160
K I E K H N H Y Q K Q L E I S H Q K L K H V E S I G D P E H I S R

                    170                 180                 190
N K E K Y V L L E E K T K E L G Y K V K K H L Q D L S S R V S R A

200
R H N E L
```

Using standard recombinant techniques, a chemically

synthesized gene encoding this rat protein may be prepared. The

chemically synthesized gene comprises a chemically synthesized

polynucleotide which codes on expression for the amino acid

25 sequence of the rat protein given above.

A method of thrombolysis in a mammal is also provided.

In accordance with the invention, the method utilizes the t-PA

hepatic clearance-inhibiting effect of the 39kDa protein and

fragments thereof. The method comprises administering to the

30 mammal, preferably intravenously, a thrombolytically effective

amount of t-PA and a t-PA-hepatic clearance-inhibiting effective

amount of the 39kDa protein or a t-PA-hepatic clearance-

inhibiting fragment thereof. The preferred amount of t-PA is
between 0.15 and 1.5 mg/kg of body weight/dose. The preferred
amount of 39kDa protein or fragment administered to the mammal
is between 60 to 6,000 mg/kg of body weight/dose. When the

5   fragment of the 39kDa protein is the 28kDa protein, the
preferred amount administered to the mammal is between 38 to
3,800 mg/kg of body weight/dose. The 39kDa protein or fragment
thereof may be administered to the mammal up to 20 minutes prior
to administering the t-PA.

10   A composition for treating thrombolytic diseases which
may be employed in the method of thrombolysis includes an
effective amount of t-PA and an effective amount of a t-PA
hepatic clearance-inhibiting 39kDa protein or a t-PA hepatic
clearance-inhibiting fragment thereof. The t-PA is preferably

15   present in a dosage amount of from about 0.15 to 1.5 mg/kg of
body weight/dose. The 39kDa protein or fragment thereof is
present in a dosage amount of from about 60 to 6,000 mg/kg of
body weight/dose. When the fragment of the 39kDa protein is the
28kDa protein, the dosage amount is from about 38 to 3,800 mg/kg

20   of body weight/dose.

          t-PA is primarily used in the treatment of
thrombolytic diseases, but t-PA has also been used for treating
myocardial infarctions, tumors via fibrinolysis, and sickle cell
anemia via fibrinolysis. The present invention accordingly

25   provides a pharmaceutical composition for a mammalian patient
containing t-PA, the improvement comprising further including a
t-PA hepatic clearance-inhibiting amount of 39kDa protein or a
t-PA hepatic clearance-inhibiting fragment thereof. The t-PA is

preferably present in a dosage amount of from about 0.15 to 1.5

mg/kg of body weight/dose. The 39kDa protein or fragments

thereof is present in a dosage amount of from about 60 to 6,000

mg/kg of body weight/dose. When the fragment of the 39kDa

5   protein is the 28kDa protein, the dosage amount is from about 38

to 3,800 mg/kg of body weight/dose.

The ability of the 39kDa protein to bind to the t-PA

hepatic receptor is demonstrated in the following examples.

<div align="center">EXAMPLE 3</div>

10   **Specific Binding of the 39kDa Protein to the MH_1C_1 Cell surfac**

Rat MH_1C_1 hepatoma cells are known to specifically bind

^{125}I-t-PA with high affinity to the cell surface (K_d = 4.9 ± 1.3

nM (SD); Bmax = 78,000 ± 35,000 (SD)) (Bu, G. et al., J. Biol.

Chem. 267, 15595-15602). A specific interaction between the

15   39kDa protein and rat MH_1C_1 hepatoma cells was demonstrated by

the following saturation binding analyses.

Cell Culture and Media

Rat MH_1C_1 hepatoma cells were grown in Eagle's minimum

essential media (MEM) with Earle's salts (Gibco BRL)

20   supplemented with 10% fetal calf serum, penicillin (100 U/ml),

and streptomycin (100 μg/ml) at 37°C in humidified air

containing 5% CO_2 as described in Bu, G. et al., (1992) J. Biol.

Chem. 267, 15595-15602. Cell monolayers were used at

approximately 90% confluence, and the media was replaced the day

25   prior to each experiment.

Radiolabelling of purified proteins

Recombinant 39kDa protein and t-PA were radiolabelled

with carrier-free sodium ^{125}I-iodide (DuPont New England Nuclear

Products) using the Iodogen procedure described in Bu, G. et
al., (1992) J. Biol. Chem. 267, 15595-15602. Specific
radioactivities for each radiolabelled protein were between 1
and 2 x 10^7 cpm/μg of protein as measured by γ scintillation
5   spectrometry.

Saturation Binding Analysis

Cells were grown in 12 well dishes to approximately 10^6
cells per well. Monolayers were taken directly from 37°C and
cooled on ice. The binding buffer used for the recombinant
10   39kDa protein was PBSc (phosphate-buffered saline supplemented
with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$). Each cell monolayer was
washed three times with PBSc prior to the addition of binding
buffer containing various concentrations of radiolabelled
^{125}I-39kDa protein, either in the presence or absence of an excess
15   of unlabelled protein. Cell monolayers were incubated at 4°C
with between 1 and 24 nM recombinant ^{125}I-39kDa protein, either in
the presence or absence of 0.5 μM unlabelled protein.

Following a 90 minute incubation, cell monolayers were
washed three additional times with PBSc to remove non-
20   specifically associated ligand, and lysed in "low SDS lysis
buffer" (0.0625 M Tris-HCl, pH 6.8, with 0.2% sodium dodecyl
sulfate, 10% glycerol, and 0.01% bromophenol blue), as described
in Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602. The
amount of cell associated ligand was determined by γ
25   scintillation spectrometry. In cases where t-PA and the 39kDa
protein were co-bound, the t-PA binding buffer (PBSa containing
0.2 mM $CaCl_2$ and 10 mM ϵ-amino-n-caproic acid) was used as

described in Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602.

As shown in FIG. 1A, non-specific binding of ^{125}I-39kDa protein increased linearly over the range of concentrations

5   tested, while total binding increased in a curvilinear fashion, approaching an asymptote above 12 nM. Scatchard analysis (23) (inset to FIG. 1A) is consistent with a single homogeneous population of binding sites and yields an equilibrium dissociation constant (K_d) of 3.2 nM with 220,000 binding sites

10   per cell, for this particular experiment.

The results from ten independent binding experiments indicate a mean K_d value of 3.3 ± 0.9 (SD) nM for ^{125}I-39kDa protein binding to MH_1C_1 cells, with an average of 380,000 ± 190,000 (SD) binding sites per cell, as shown in the following

15   table, Table 1. A represents the mean values with standard deviations for binding of both the 39kDa protein and t-PA to MH_1C_1 cells. The cumulative data from all independent binding experiments was used. B represents the mean values with standard deviation from only simultaneously performed binding

20   experiments. C represents data derived from simultaneous binding experiments performed using either untreated MH_1C_1 cells, or cells pre-incubated for 30 minutes with 0.1% saponin at 4°C.

Tabl 1

8p cific Binding of ^{125}I-39kDa Pr tein and ^{125}I-t-PA to MH$_1$C$_1$ Cells

ligand	preincubation	Bmax ± S.D. (10^3 sites/cell)	Kd ± S.D. (nM)	Experiment (n)
A. Summary				
^{125}I-39kDa	none	380 ± 190	3.3 ± 0.9	10
^{125}I-t-PA	none	78 ± 35	4.9 ± 1.3	7
B. Simultaneous Assays				
^{125}I-39kDa	none	390 ± 150	3.5 ± 0.9	4
^{125}I-t-PA	none	60 ± 35	5.6 ± 1.5	4
C. Simultaneous Assays Performed in the Presence or Absence of Saponin				
^{125}I-39kDa	none	250 ± 8	2.7 ± 1.3	3
^{125}I-t-PA	saponin	515 ± 24	9.1 ± 3.6	3

These data indicate both specific and saturable
binding of the 39kDa protein to a single kinetic species of
high affinity receptor on the MH$_1$C$_1$ cell surface.

The nature of the 39kDa protein binding species was
shown by visualizing cell lysates from each binding experiment
by sodium dodecyl sulfate polyacrylamide gel electrophoresis
(SDS-PAGE) (FIG. 1B). Under the mild conditions used in these
experiments (0.2% SDS), the 39kDa protein appeared to bind in
isolation, without apparent association with any auxiliary
binding proteins. These experiments demonstrate that there is
a specific and saturable interaction between the 39kDa protein
and an MH$_1$C$_1$ cell surface molecule(s).

EXAMPLE 4

Specific Binding of ^{125}I-t-PA and ^{125}I-39kDa Protein t HepG2 Cells

The following saturation binding analyses

demonstrated that there is a specific interaction between the

39kDa protein and human hepatoma HepG2 cells. Additionally,

the binding parameters to human hepatoma HepG2 cells of ^{125}I-

t-PA and ^{125}I-39kDa protein were compared by parallel saturation

binding experiments.

Binding studies were carried out at 4°C to avoid

possible ligand uptake and degradation. Binding buffer for

^{125}I-t-PA included 10 mM EACA since this lysine analog reduced

nonspecific ^{125}I-t-PA binding (presumably to lysine residues on

the cell surface) without affecting specific ^{125}I-t-PA binding.

(See, Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602).

Throughout this example, protein concentration was determined

using a Bio-Rad protein assay with bovine serum albumin as a

standard. Cell number was determined by counting cells with a

hemocytometer.

Cell Culture

Human hepatoma HepG2 cells were maintained in

culture as described in (Schwartz, A. L. et al., (1981) J.

Biol. Chem. 256, 8878-8881). Cells were cultured in Earle's

minimum essential medium (MEM) with glutamine (Gibco

Laboratories) supplemented with 10% (v/v) fetal calf serum

(Gibco Laboratories), penicillin (100 units/ml), streptomycin

(100 μg/ml), and were incubated at 37°C in humidified air

containing 5% CO_2. Cultures were supplemented with fresh media

12 hours prior to use. Cell monolayers were generally
cultured for two days before use at 80-90% confluence.

Production and Isolation of 39kDa Protein

         The 39kDa protein was produced and isolated in
5   accordance with the procedure of Example 1.

Protein Iodination

         Iodination of t-PA and 39kDa protein were performed
using IODOGEN (Pierce Chemical Co.) as described in Bu, G. et
al., (1992) J. Biol. Chem. 267, 15595-15602. The specific
10   activity was generally 5-10 μCi/μg of protein as determined by
γ scintillation spectrometry. The unincorporated ^{125}I after
gel-filtration purification over a PD-10 column (Pharmacia)
was less than 2% of the total radioactivity.

Saturation Binding Assays

15   Cells were seeded into multiwell (12 wells/plate)
disposable plastic tissue culture plates 2 days prior to
assay. Ligand binding buffer for t-PA was composed of
phosphate-buffered saline (PBS) supplemented with 0.2 mM $CaCl_2$
and 10 mM EACA, whereas PBSc (PBS containing 1 mM $CaCl_2$ and 0.5
20   mM $MgCl_2$) was used for 39kDa protein binding. Binding
experiments were performed at 4°C to prevent possible
concomitant internalization during the binding interval. Cell
monolayers were washed three times on ice with prechilled
binding buffer. Binding was initiated by adding 0.5 ml
25   binding buffer containing selected concentrations of [125]I-
labelled ligand in the absence or presence of an excess
unlabelled ligand (1 μM). After incubation at 4°C for 1.5
hours, buffer containing unbound ligand was removed. Cells

were then washed three times with binding buffer and lysed in

0.0625 M Tris-HCl, pH 6.8, containing 0.2% (w/v) SDS and 10%

(v/v) glycerol ("low-SDS lysis buffer"). Radioactivity of

cell lysates was quantified by γ scintillation spectrometry.

5   For some experiments, cell lysates were further analyzed by

SDS-PAGE and autoradiography as described below. Total

binding was determined in the presence of ^{125}I-labelled ligand

alone. Nonspecific binding was determined in the presence of

excess unlabelled ligand. Specific ligand binding was defined

10   as the difference between total and nonspecific binding.

        Specific binding of ^{125}I-t-PA over the concentration

range of 0.1-48 nM yielded a curvilinear plot. FIG. 2A shows

a representative experiment from seven such experiments

performed. Scatchard analysis of the binding data showed a

15   curvilinear plot concave upwards (*inset*, FIG. 2A). This

result may be due to either the presence of multiple classes

of binding sites with different but fixed affinities, or the

existence of site-site interactions of the type defined as

"negative cooperativity". (See Meyts, P. D. et al., (1976) J.

20   Biol. Chem. 251, 1877-1888). Due to the type of saturation

binding experiments performed, these two possibilities could

not be distinguished. When multiple classes of binding sites

are assumed, an estimate of two binding sites best fit the

data. From the Scatchard analysis shown in FIG. 2A, 40,300

25   sites are estimated for the high affinity binding (K_d = 1.6 nM)

and 146,200 sites (K_d = 32.5 nM) for the low affinity binding.

When cell lysat s from selected ligand concentrations were

analyzed via SDS-PAGE, specific binding occurred predominantly

in the form of the ^{125}I-t-PA:PAI-1 complex. This is consistent

with PAI-1-dependent ^{125}I-t-PA binding on HepG2 cells. (See,

Morton, P. A. et al., (1989) J. Biol. Chem. 264, 7228-7235;

Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602).

5   Specific binding of ^{125}I-39kDa protein to HepG2 cells

was performed over the concentration range of 1-24 nM. As

shown in FIG. 2B, the 39kDa protein specifically bound to

HepG2 cells with a low level of non-specific binding.

Saturation of specific binding was observed at ^{125}I-39kDa

10   protein concentrations in excess of 12 nM. Scatchard analysis

of the binding data from five such experiments yielded 197,000

± 23,000 (S.D.) homogeneous high affinity surface binding

sites per cell with an apparent K_d = 5.1 ± 0.8 nM (S.D.)

(166,000 binding sites per cell and K_d = 4.9 nM for the

15   experiment shown in FIG. 2B). When post-binding cell lysates

were analyzed by SDS-PAGE, ^{125}I-39kDa protein was observed at

its native molecular mass (i.e., 39kDa) indicating that the

binding did not include an SDS-stable complex with other

proteins.

20   *   *   *   *   *

          The following example demonstrates that low density

lipoprotein receptor related protein (LRP) is a receptor for

the 39kDa protein.

EXAMPLE 5

25   **Cross-linking of ^{125}I-39kDa Protein to the MH$_1$C$_1$ Cell Surface**

          The 39kDa protein was originally isolated via its

ability to co-purify with a low density lipoprotein receptor

related protein (LRP) by α2-macroglobulin affinity

chromatography. See Ashcom, J.D. et al., (1990) J. Cell
Biol. 110, 1041-1048; Jensen, P.H. et al., (1989) FEBS Lett.
255, 275-280. This low density lipoprotein receptor-related
protein (LRP) with a molecular mass of >500 kDa has recently

5   been identified and characterized as a new member of the LDL
receptor family (Herz, J. et al., (1988) EMBO J. 7, 4119-4127;
Beisiegel, U. et al., (1989) Nature 341, 162-164; Brown, M.S.
et al., (1991) Curr. Opin. Lipidol. 2, 65-72). This plasma
membrane receptor is unique not only because of its large

10 molecular size but also because of its multifunctional nature
in ligand recognition. Studies have shown that LRP mediates
binding and endocytosis of both apoprotein E-enriched
β-migrating very low density lipoprotein (β-VLDL) (Beisiegel,
U. et al., (1989) Nature 341, 162-164; Lund, H. et al., (1989)

15 Proc. Natl. Acad. Sci. USA 86, 9318-9322) and protease- or
methylamine-activated α_2-macroglobulin (α_2M) (Strickland, D.K.
et al., (1990) J. Biol. Chem. 265, 17401-17404). Based on the
following cross-linking studies, it was found that LRP
functions as a MH_1C_1 cell specific 39kDa protein receptor.

20 Metabolic Labelling

        Cells were grown in 10 cm dishes as described above in
Example 3. Cell monolayers were washed three times with
pre-warmed Eagle's minimum essential media (MEM) with Earle's
salts lacking L-methionine (Washington University Center for

25 Basic Cancer Research). Following two 15 minute
pre-incubations at 37°C with this same media, cells were
labelled by the addition of methionine deficient MEM
containing 0.4 mCi/ml ^{35}S-methionine (Amersham Corp.).

Labelling was carried out for 4-5 hours at 37°C. After

metabolic labelling, cell monolayers were cooled on ice,

washed three times with cold PBSc, and incubated with 14 nM of

the radiolabelled ^{125}I-recombinant 39kDa protein of Example 3,

5   either in the presence or absence of an excess of unlabelled

39kDa protein, to allow specific binding to the cell surface

receptor.

Chemical Cross-Linking

        Saturation binding was performed as described above, but

10   with 10 cm dishes of MH$_1$C$_1$ cells. Either unlabelled or

^{35}S-methionine labelled cells were used. Following incubation

with radiolabelled and unlabelled ligands (39kDa proteins),

cell monolayers were washed three times with PBSc to remove

non-specifically associated ligand and incubated with either

15   PBSc alone or PBSc containing 0.5-1 mM

dithio*bis*(sulfosuccinnimidylpropionate) (DTSSP) (Pierce

Chemical Co.), a disulfide bond containing, thiocleavable

cross-linker. After one hour, cross-linking reactions were

quenched by three washes with tris-buffered saline, and cell

20   monolayers were solubilized in PBSa containing 1% Triton X-100

and 1 mM PMSF. Immunoprecipitations were performed as

described below.

Immunoprecipitation

        Aliquots of cell lysates from cross-linking and metabolic

25   labelling experiments were added to equal volumes of PBSc

containing 1% Triton X-100, 0.5% sodium deoxycholate, 1%

sodium dodecyl sulfate (SDS), 0.5% bovine serum albumin , and

1mM PMSF (immunomix). When metabolically labelled cells were

used, lysates were first pre-cleared by an overnight

incubation with rabbit pre-immune serum and a subsequent

incubation with an excess of protein-A beads (Repligen).

Samples were then immunoprecipitated with 0.6 μg of affinity

5   purified α-39kDa antibody or affinity purified rabbit α-human

LRP antibody (American Red Cross, Rockville, MD). Affinity

purified α-39kDa antibody was prepared in New Zealand white

rabbits following injection of purified recombinant 39kDa

protein in accordance with the procedure described in

10   Schwartz, A.L. et al., (1983) J. Biol. Chem. 258, 11249-11255.

When unlabelled cells were cross-linked to a single

radiolabelled ligand, no pre-immune clearance of cell lysates

was necessary, and either α-LRP immune serum or α-39kDa

protein immune serum was substituted for the affinity purified

15   antibody. In some cases, samples of cell lysates were also

immunoprecipitated with 30 ml of immune α-t-PA serum.

Following each immunoprecipitation, bead samples were split in

half and boiled in 0.0625 M Tris-HCl, pH 6.8, 2% SDS, and 10%

glycerol as described in Laemmli, U.K., (1970) Nature 227,

20   680-685, either in the presence or absence of 5%

2-mercaptoethanol. Samples were then separated by sodium

dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE),

with the gels subsequently fixed and vacuum dried.

Autoradiography was performed at -70°C with Hyperfilm-MP

25   autoradiography film (Amersham Corp.).

FIG. 3 shows the results from a typical experiment.

Under conditions where no cross-linker was used (FIG. 3C and

D), a single band corresponding to the 39kDa protein

(arrowhead) was immunoprecipitated only with α-39kDa serum. Samples lectrophoresed under either reducing or non-reducing conditions show that the α-LRP antibody was unable to immunoprecipitate the radiolabelled 39kDa ligand in the

5   absence of cross-linker. Since there was a marked decrease in the amount of ^{125}I-39kDa protein bound in the presence of an excess of unlabelled protein, the association of ligand with its cell surface receptor was shown to be specific. In FIG. 3A and 3B, the radiolabelled 39kDa protein was both bound and

10   cross-linked to the MH_1C_1 cell surface. When samples from each immunoprecipitation were analyzed under non-reducing conditions (FIG. 3A), the complex of ligand associated radioactivity was immunoprecipitable with both α-LRP and α-39kDa antibodies. This complex was of very high apparent

15   molecular weight, remaining largely in the stacking gel during electrophoresis (arrows). Again, this interaction was shown to be specific, as the association of ^{125}I-39kDa with this high molecular weight complex was abrogated in the presence of an excess of unlabelled 39kDa protein. When these same samples

20   were electrophoresed in the presence of 5% 2-mercaptoethanol to dissociate the cross-linker (FIG. 3B), a single 39kDa binding species was seen.

         The foregoing experiments demonstrate that there is a specific interaction between the 39kDa protein and the MH_1C_1

25   cell surface which is mediated, at least in part, by an association with LRP.

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EXAMPLE 6

Chemical Cross-linking of t-PA:PAI-1
Complex to LRP on HepG2 Cells

Studies by Orth, K. et al., (1992) Proc. Natl. Acad.

5   Sci. USA 89, 7422-7426, demonstrated via ligand blotting the

binding of t-PA:PAI-1 complexes to isolated LRP immobilized on

gels. Endocytosis by LRP and subsequent degradation of

t-PA:PAI-1 complexes was also observed in simian COS tumor

cells, a non-hepatocyte cell line. The following experiment

10   showed that a direct interaction between the t-PA:PAI-1

complex and LRP in human hepatocytes occurs based on chemical

cross-linking following ligand binding to human hepatoma HepG2

cells. In accordance with the present invention, it was

hypothesized that binding and subsequent cross-linking of the

15   t-PA:PAI-1 complex to LRP on HepG2 cells should yield a

trimeric complex, t-PA:PAI-1-LRP. Therefore, if one component

in this trimeric complex is radiolabelled, antibodies to the

other components should be able to immunoprecipitate the

radiolabelled protein. A thio-cleavable, water-soluble, and

20   membrane-impermeant reagent DTSSP was used as the cross-

linker, as described in Bu, G. et al., (1992) J. Biol. Chem.

267, 15595-15602; and Bu, G. et al., (1992) Proc. Natl. Acad.

Sci. USA 89, 7427-7431. Following ligand binding and cross-

linking, cell lysates were used for immunoprecipitation with

25   various antibodies, in order to characterize the nature of the

cross-linked protein complexes. Chemical cross-linking

experiments were performed with either ^{125}I-t-PA cross-linked to

unlabelled HepG2 cells, or unlabelled t-PA cross-linked to ^{35}S-

methionine metabolically-labelled HepG2 cells. Throughout

this example, protein concentration was determined using a
Bio-Rad protein assay with bovine serum albumin as a standard.
Cell number was determined by counting cells with a
hemocytometer.

5   Metabolic Labelling

       Cells growing in 10 cm dishes at about 80%
confluence were incubated for 30 min at 37°C in two changes of
Earle's minimum essential medium lacking L-methionine and
containing 2 mM L-glutamine (Gibco Laboratories). Metabolic
10   labelling was initiated by the addition of the above medium
supplemented with ^{35}S-methionine (400 μCi/ml)(Amersham).
Following incubation for 5 hours at 37°C, cell monolayers were
washed with binding buffer and were used for ligand binding
and chemical cross-linking experiments as described below.

15   Chemical Cross-linking

       Experiments were performed with either ^{125}I-labelled
ligand cross-linked to unlabelled cells or unlabelled ligand
cross-linked to ^{35}S-methionine metabolically-labelled cells.
After ligand binding at 4°C, each cell monolayer was washed
20   three times with PBSc. Chemical cross-linking was performed
by incubating the cell monolayer with PBSc containing 0.5 mM
dithio*bis*(sulfosuccinnimidylpropionate) (DTSSP) (Pierce
Chemical Co.). After 30 min at 4°C, the reaction was quenched
by washing the cell monolayer two times with Tris-buffered
25   saline (TBS). Cells were then solubilized in PBSc containing
1% (v/v) Triton X-100 (Sigma Chemical Co.) and 1 mM
phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co.)
("lysis buffer") for 30 min at 4°C with a brief sonication at

low power output and occasional vortexing. These solubilized cell lysates were subsequently used for immunoprecipitation.

Antibodies

All antibodies used in these studies were rabbit polyclonal antibodies. Anti-t-PA, anti-PAI-1, and anti-39kDa protein antibodies were produced using recombinant proteins. Anti-LRP antibody was generated against purified human placental LRP provided by Dudley K. Strickland (American Red Cross, Rockville, Maryland). Total IgG for each antibody was purified using Protein A-agarose. Specific IgG for each antibody was affinity-purified against its corresponding protein coupled to CNBr-activated Sepharose 4B (Pharmacia).

Immunoprecipitation

Each cell lysate from chemical cross-linking experiments was divided into equal portions depending upon the number of antibodies used. Each aliquot of the cell lysate was then brought to 0.5 ml with lysis buffer and was mixed with 0.5 ml of PBSc containing 1% (v/v) Triton X-100 (Sigma Chemical Co.), 0.5% (w/v) sodium deoxycholate (Sigma Chemical Co.), 1% (w/v) SDS, 0.5% (w/v) bovine serum albumin (Sigma Chemical Co.), and 1 mM PMSF (Sigma Chemical Co.) ("immunomix"). SDS was included in the immunomix to reduce non-specific immunoprecipitation. Primary antibody (10 μl normal rabbit serum (Sigma Chemical Co.), or 1 μg affinity-purified IgG) was added, and the samples were rocked overnight at 4°C followed by incubation for 1 hour at room temperature with 50 μl of protein A-agarose (Repligen). Non-bound radioactivity was removed by washing protein A-agarose beads

three times with immunomix and three times with PBSc. The

protein A-agarose beads were divided into two equal parts

before the final wash for nonreducing and reducing assays. The

immunoprecipitated material was then released from the beads

5   by boiling each sample for 5 min in 0.0625 M Tris-HCl, pH 6.8,

2% (w/v) SDS, and 10% (v/v) glycerol ("Laemmli sample buffer")

(Laemmli, U. K., (1970) Nature 227, 680-685) with or without

5% (v/v) 2-mercaptoethanol, and was analyzed by SDS-PAGE as

described below.

10   SDS-PAGE and Autoradiography

         Samples of cell lysates or immunoprecipitations were

analyzed by SDS-PAGE using polyacrylamide slab gels as

described in Laemmli, U. K., (1970) Nature 227, 680-685, under

reducing or non-reducing conditions. The following pre-

15   stained molecular weight standards from Bio-Rad were used:

myosin: 205 kDa; β-galactosidase: 117 kDa; bovine serum

albumin: 80 kDa; ovalbumin: 50 kDa. Autoradiography of [125]I-

labelled proteins was performed with dried polyacrylamide gels

using Hyperfilm-MP (Amersham). For fluorography of [35]S-

20   labelled proteins, gels were impregnated with Amplify

(Amersham), dried, and exposed to films. Films were placed at

-70°C for various periods of time as specified in each figure

prior to developing.

         FIG. 4 shows an experiment with ^{125}I-t-PA binding and

25   cross-linking to HepG2 cells. Cell lysates without or with

cross-linking were immunoprecipitated with one of the

following antibodies: normal rabbit serum, anti-t-PA antibody,

anti-PAI-1 antibody, or anti-LRP antibody. The

immunoprecipitated material was then analyzed by SDS-PAGE

under nonreducing (FIG. 4A) or reducing (FIG. 4B) conditions.

Under nonreducing conditions and without cross-linking, the

^{125}I-t-PA:PAI-1 complex was immunoprecipitated by both anti-t-PA

5   (lane 2) and anti-PAI-1 antibody (lane 3) confirming the PAI-

1-dependent ^{125}I-t-PA binding to HepG2 cells with the ^{125}I-

t-PA:PAI-1 complex being the vast majority of the bound

ligand. Uncomplexed ^{125}I-t-PA was observed with anti-t-PA

antibody (lane 2) but not with anti-PAI-1 antibody (lane 3).

10   The appearance of the uncomplexed ^{125}I-t-PA (about 40% of the

total cell-associated ligand radioactivity) in post-binding

lysates was predominantly due to dissociation of ^{125}I-t-PA and

PAI-1 during immunoprecipitation and washing with the

immunomix containing 1% SDS and 1% Triton X-100. When the

15   cell lysates were analyzed directly using low-SDS sample

buffer without prior immunoprecipitation, more than 90% of the

specific binding species was in the form of ^{125}I-t-PA:PAI-1

complex as appears in FIG. 5B (data not shown). In the

absence of cross-linking, anti-LRP antibody did not

20   immunoprecipitate any ^{125}I-labelled ligand (lane 4) indicating

that this antibody does not have any cross-reactivity with the

^{125}I-t-PA:PAI-1 complex. However, with chemical cross-linking,

anti-LRP antibody (lane 8), in addition to anti-t-PA antibody

(lane 6) and anti-PAI-1 antibody (lane 7), immunoprecipitated

25   ^{125}I-labelled ligands. Since anti-LRP antibody does not cross-

react with the ^{125}I-t-PA:PAI-1 complex (lane 4), this specific

immunoprecipitation by anti-LRP antibody must be a result of

the ^{125}I-t-PA:PAI-1 complexes cross-linked to LRP. The ligand-

receptor complexes consisting of ^{125}I-t-PA:PAI-1 and LRP
appeared as a broad smear migrating on top of a 7% SDS-
polyacrylamide gel with a molecular mass estimated to be
greater than 600 kDa. However, when the same material was

5   analyzed by SDS-PAGE under reducing conditions, the
radioligand ^{125}I-t-PA:PAI-1 complex and ^{125}I-t-PA appeared
following reduction of the thiocleavable cross-linker DTSSP
(FIG. 4B). Reduction of the cross-linked material caused
additional dissociation of the ^{125}I-t-PA:PAI-1 complex resulting

10   in an even greater amount of free ^{125}I-t-PA. Normal rabbit
serum showed no specific interaction with any of the cross-
linked material (lanes 1 and 5).

        To exclude the possibility that chemical cross-
linking of ^{125}I-t-PA:PAI-1 to HepG2 cells generated an antigenic

15   epitope for anti-LRP antibody, unlabelled t-PA was cross-
linked to ^{35}S-methionine metabolically-labelled HepG2 cells.
This approach allowed direct visualization of the binding
protein for the t-PA:PAI-1 complex or the 39kDa protein and
the determination of its relationship to LRP. Following

20   ^{35}S-methionine metabolic labeling of HepG2 cells, cell
monolayers were incubated with binding buffer alone, or
binding buffer containing t-PA (15 nM) or the 39kDa protein
(15 nM) (FIG. 6). Cell monolayers in the absence of ligand
was lysed directly without chemical cross-linking whereas

25   those following ligand binding were subjected to chemical
cross-linking with DTSSP. Each of the cell lysate
preparations was then immunoprecipitated with one of the
following antibodies: normal rabbit serum (lanes 1, 6, 11),

40

anti-t-PA antibody (lanes 2, 7, 12), anti-PAI-1 antibody

(lanes 3, 8, 13), anti-39kDa protein antibody (lanes 4, 9,

14), or anti-LRP antibody (lanes 5, 10, 15). The

immunoprecipitated materials were then analyzed by 7% SDS-PAGE

5   under either nonreducing or reducing conditions.

As shown in FIG. 6, anti-PAI-1 antibody

immunoprecipitated PAI-1 from all the HepG2 cell lysates

consistent with the well recognized production of PAI-1 by

HepG2 hepatoma cells. (See, Bugelski, P. J. et al., (1989)

10   Thromb. Res. 53, 287-303; Owensby, D. A. et al., (1991) J.

Biol. Chem. 266, 4334-4340; Bu, G. et al., (1992) J. Biol.

Chem. 267, 15595-15602; Sprengers, E. D. et al., (1985) J. Lab

Clin. Med. 105, 751-758). When an ^{35}S-methionine-labelled

HepG2 monolayer was analyzed without incubation with either

15   t-PA or 39kDa protein, only the anti-LRP antibody was able to

immunoprecipitate the two LRP subunits (515kDa and 85kDa)

(lanes 5). However, following binding of unlabelled t-PA and

chemical cross-linking, the ^{35}S-labelled ligand-receptor

complexes were immunoprecipitable not only by anti-LRP

20   antibody (lanes 10) but also by anti-t-PA antibody (lanes 7)

and anti-PAI-1 antibody (lanes 8). When these materials were

analyzed under reducing conditions, the LRP subunits, as well

as the t-PA:PAI-1 complexes were observed. These results are

thus consistent with the nature of the high molecular weight

25   receptor-ligand complexes composed of the t-PA:PAI-1 complex

(ligand) and LRP (receptor). Radiolabelling of the t-PA:PAI-1

complex was a result of complex formation between unlabell d

t-PA and endogenous ^{35}S-methionine-labelled PAI-1.

When the ^{35}S-methionine-labelled HepG2 cell monolayer
was exposed to externally added 39kDa protein, the
^{35}S-methionine-labelled ligand-receptor complex appearing on
top of the gel was immunoprecipitable not only by anti-LRP
5   antibody (lanes 15), but also by anti-39kDa protein antibody
(lanes 14). Analysis of these cross-linked materials under
reducing conditions yielded the 515kDa radiolabelled LRP band
when immunoprecipitated with anti-39kDa antibody, further
demonstrating the receptor protein for 39kDa protein on HepG2
10   cells is LRP.

EXAMPLE 7

The 39kDa Protein is Cross-linked Predominately to LRP on the MH_1C_1 Cell Surface

It was found that LRP is the predominant 39kDa
15   protein binding species by cross-linking unlabelled 39kDa
protein to the surface of radiolabelled MH_1C_1 cells using the
procedures of Example 5 with the following modifications.
Labelling was carried out at 37°C for 4-5 hours in methionine
deficient Earle's MEM media supplemented to 0.4 mCi/ml with
20   ^{35}S-methionine (Amersham Corp.). Following several washes,
labelled monolayers were bound with unlabelled 39kDa protein,
and either cross-linked by the addition of 0.5 mM DTSSP, or
incubated in PBSc alone. Lysis and immunoprecipitation were
carried out as described above in Example 5.

25   FIG. 7 shows the results from a typical experiment.
When radiolabelled MH_1C_1 cells were cross-linked to unlabelled
39kDa protein, a complex of very high apparent molecular
weight resulted, which was immunoprecipitable with both the
α-39kDa and, to a lesser degree, the α-human LRP affinity

42

purified antibodies (FIG. 7A, lanes 4-6). Antibodies to human

LRP were used as no α-rat LRP affinity purified antibody is

presently available. This high molecular weight complex was

electrophoretically identical to that seen when radiolabelled

5   ^{125}I-39kDa protein was cross-linked to unlabelled MH_1C_1 cells

(compare with FIG. 3A, lanes 2 and 3). In addition to the

cross-linked complex, uncross-linked endogenously labelled

39kDa protein (lane 5, *arrowhead*), and both the 520kDa (*closed

arrow*) and 85kDa (*open arrow*) subunits of the LRP receptor

10   were visible. When these same immunoprecipitations were

electrophoresed in the presence of the reducing agent

2-mercaptoethanol, the cross-linker was cleaved, and the

cross-linked complex was dissociated into its component

peptides (FIG. 7B, lanes 4-6). In both cases, the

15   cross-linked complex was shown to contain almost exclusively

the 520kDa subunit, and to a lesser degree, the 85kDa subunit

of the LRP receptor. In lanes resulting from

immunoprecipitation with the α-human LRP antibody, less of the

high molecular weight cross-linked complex was evident. It is

20   believed that this occurs partly because the recognition

epitopes for the α-LRP antibody are obscured by binding of the

39kDa protein.

When metabolically labelled cell lysates which have

been bound with, but not cross-linked to, unlabelled 39kDa

25   protein were immunoprecipitated with the α-39kDa antibody,

only endogenously labelled 39kDa protein was seen (*arrowhead*).

No high molecular weight compl x was evident. Also, in the

absence of cross-linker, immunoprecipitation of metabolically

labelled cell lysates with α-human LRP antibodies results in
only the 520kDa (*closed arrow*) and the 85kDa (*open arrow*)
subunits of the LRP receptor.   Again, no high molecular weight
complex was evident.   Samples electrophoresed under both

5   reducing and non-reducing conditions gave similar results.

        This example demonstrates that the LRP is the
predominant 39kDa protein binding species on the MH_1C_1 cell
surface.

                    *   *   *   *   *

10   Example 8 demonstrates that both the 39kDa protein
and t-PA specifically co-bind to a single LRP molecule.

                          EXAMPLE 8

          t-PA and the 39kDa Protein can be Cross-linked to
          the Same LRP Receptor Complex
15
        0.5nM ^{125}I-39kDa protein and 20nM unlabelled t-PA were
co-bound to MH_1C_1 cells both in the presence and absence of an
excess of unlabelled 39kDa protein.   Following several washes
to remove non-specifically associated ligand, cell monolayers

20   were either incubated in 1mM DTSSP or in PBSc alone.   Once
cross-linked, cell monolayers were washed, lysed and
immunoprecipitated as described above.

        FIG. 8 shows the results from a typical experiment.
When the ^{125}I-39kDa protein and unlabelled t-PA were co-bound to

25   the MH_1C_1 cell surface and incubated in the absence of
cross-linker (FIG. 8C and D), the radiolabelled 39kDa protein
was immunoprecipited from cell lysates with only the α-39kDa
antibody (*arrowhead*).   Neither the α-LRP antibody nor the
α-t-PA antibody showed any affinity for the radiolabelled

30   39kDa ligand.   Samples electrophoresed under both reducing and

non-reducing conditions gave similar results. As in Example

3, interaction of the 39kDa protein with the MH_1C_1 cell surface

was shown to be specific by abrogation with an excess of

unlabelled 39kDa protein. When MH_1C_1 cells were pre-bound with

5   ^{125}I-39kDa protein and unlabelled t-PA, and incubated in the

presence of cross-linker (FIG. 8A and B), a high molecular

weight complex of ligand-associated radioactivity resulted,

which was immunoprecipitable with α-39kDa, α-t-PA, and α-LRP

antibodies. Each of these interactions was specific, as each

10   was abolished in samples incubated in the presence of an

excess of unlabelled 39kDa protein. This protein complex of

^{125}I-39kDa, t-PA, and LRP was of very high apparent molecular

weight as before, remaining largely in the stacking gel during

electrophoresis (*arrows*). When these same samples were

15   electrophoresed in the presence of 2-mercaptoethanol to cleave

the cross-linker, each resulted in a single radiolabelled band

corresponding to the exogenously added ^{125}I-39kDa protein

(*arrowhead*).

In order to determine if cross-linking of the 39kDa

20   protein to the MH_1C_1 cell surface generates an epitope for

recognition by the α-t-PA antibody, similar experiments were

performed with ^{125}I-39kDa protein bound and cross-linked to the

MH_1C_1 cell surface, but in the absence of exogenously added,

unlabelled t-PA (FIG. 8E and F). In this case, the high

25   molecular weight complex of ligand-associated radioactivity

was immunoprecipitable only with α-LRP and α-39kDa antibodies,

and not with the α-t-PA antibody.

45

This experiment shows that the simple cross-linking of radiolabelled 39kDa protein to the MH_1C_1 cell surface does not change its specificity for either the α-t-PA or α-39kDa antibodies. A number of additional co-binding and

5   cross-linking experiments, performed using ^{125}I-t-PA and unlabelled 39kDa protein, gave similar results (data not shown). This data taken together with the data presented above leads to the conclusion that the 39kDa protein and t-PA can bind simultaneously to LRP on the MH_1C_1 cell surface.

10   *   *   *   *   *

It was found from the following examples that endocytosis of the 39kDa protein is receptor-mediated like t-PA and that the 39kDa protein modulates the receptor- mediated endocytosis of t-PA.

15   EXAMPLE 9

Rapid Uptake and Degradation of ^{125}I-39kDa by MH_1C_1 Cells

It is known that MH_1C_1 cells rapidly endocytose and degrade t-PA (Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602). The following single cycle uptake and

20   degradation experiments demonstrate that MH_1C_1 cells similarly endocytose the 39kDa protein.

Endocytosis experiments were performed according to Ciechanover et al., (1983) J. Biol. Chem. 258, 9681-9689 and Owensby et al., (1988) J. Biol. Chem. 263, 10587-10594. 6

25   well dishes containing 10^6 cells/well were preincubated at 4°C with 14 nM ^{125}I-39kDa protein to allow binding. Following three washes with PBSc (4°C) to remove non-specifically associated ligand, each dish was incubated at 37°C, and a pre-warmed

solution of 200 nM unlabelled 39kDa protein was added to
initiate internalization. At each time point, over a period
of 60 minutes, one of the dishes was cooled directly on ice,
and the overlying media was removed. Precipitation of the
5   overlying media was carried out by the addition of bovine
serum albumin to 10 mg/ml and trichloroacetic acid (TCA) to
20%. Both the TCA soluble (i.e., extracellular degraded
ligand) and TCA insoluble (i.e., extracellular dissociated
ligand) fractions were counted by γ scintillation
10   spectrometry.

        In addition, cell monolayers were washed three times
with PBSc and incubated 30 minutes at 4°C with a solution of
PBSc containing 0.25% Pronase (Calbiochem) to digest membrane-
associated ligand. Cells were subsequently removed from each
15   well and pelleted by centrifugation at 12,000 g.   Both the
cell pellets and the supernatants following Pronase treatment
were counted by γ scintillation spectrometry to determine both
cell associated (i.e., protease resistant) and membrane-
associated (i.e., protease sensitive) reactivity.

20   FIG. 9 shows the results from a typical experiment.
As expected, all ligand-associated radioactivity was initially
located at the cell surface. Within 3 minutes of warm-up,
however, 80% of this population became intracellular (protease
resistant) or dissociated into the media as TCA insoluble
25   (undegraded ligand) counts. The intracellular levels of
ligand reached a peak value of 50% of total at approximately
10 minut s. Concurrently, the TCA soluble radioactivity,
representing internalized and subsequently degraded ligand,

appeared in the overlying media initially at 10 minutes, with

plateau levels reached at between 45 and 60 minutes. This

kinetic pattern of uptake and degradation of a single cohort

of pre-bound ^{125}I-39kDa ligand was identical to that observed

5   for ^{125}I-t-PA with these MH$_1$C$_1$ cells.

EXAMPLE 10

Inhibition of ^{125}I-t-PA Uptake and Degradation by the 39kDa Protein on HepG2 Cells

The receptor-mediated endocytosis of the t-PA:PAI-1

10   complex by HepG2 cells has been previously demonstrated.

(See, Owensby, D. A. et al., (1988) J. Biol. Chem. 263, 10587-

10594; Morton, P. A. et al., (1989) J. Biol. Chem. 264, 7228-

7235; Underhill, D. M. et al., (1992) Blood 80, 2746-2754).

It was found from the following experiment that the

15   endocytosis and degradation of the t-PA:PAI-1 complex by HepG2

cells were also via LRP and that the 39kDa protein inhibits

the endocytosis and degradation of ^{125}I-t-PA.

Throughout this example, protein concentration was

determined using a Bio-Rad protein assay with bovine serum

20   albumin as a standard. Cell number was determined by counting

cells with a hemocytometer.

Ligand Uptake and Degradation

HepG2 cell monolayers seeded in 6-well dishes were

incubated at 37°C with 3 nM ^{125}I-t-PA in the absence or presence

25   of various competitors at selected concentrations. After

selected intervals, binding buffer overlying cell monolayers

was collected, cell monolayers were chilled on ice and washed

with prechilled binding buffer for three times to prevent

further ligand uptake and degradation. Cellular degradation

of radiolabelled ligand was determined by measuring the
appearance of TCA-soluble radioactivity in the overlying
buffer, whereas cell-associated radioactivity was determined
by lysing the cell monolayers in the low-SDS lysis buffer and

5   measuring the total radioactivity in the cell lysates. Cell-
mediated degradation of ^{125}I-t-PA was monitored in the absence
or presence of an excess of one of the following competitor
proteins: t-PA, 39kDa protein, or BSA.

The results shown in FIG. 10A demonstrate that, in

10   the absence of any competitor protein, the degradation of ^{125}I-
t-PA (initial concentration=3 nM) increased almost linearly
for at least 4 hours. This observation suggests the existence
of an intracellular receptor pool and recycling of the t-PA
receptors. (See, Owensby, D. A. et al., (1988) J. Biol. Chem.

15   263, 10587-10594; Morton, P. A. et al., (1989) J. Biol. Chem.
264, 7228-7235). Excess unlabelled t-PA (1 μM) reduced the
degradation of ^{125}I-t-PA to about 10% which represents the
nonspecific degradation of the ligand. When the 39kDa protein
was included in the incubation, specific degradation of ^{125}I-

20   t-PA was inhibited by about 75%. This is consistent with the
majority of the specific degradation occurring via LRP. This
degree of inhibition of ^{125}I-t-PA degradation by the 39kDa
protein is similar to the degree of inhibition of ^{125}I-t-PA
binding by the 39kDa protein (see FIG. 5). The control

25   protein BSA had no effect on ^{125}I-t-PA degradation.

To confirm that the inhibition of ^{125}I-t-PA
degradation by the 39kDa protein occurs at the step of ligand
uptake rather than intracellular degradation, the cell-

associated radioactivity was quantitated during the first hour

of endocytosis. As shown in FIG. 10B, about 70% of the

specific ^{125}I-t-PA uptake was inhibited by the 39kDa protein,

whereas the control protein BSA showed no inhibitory effect on

5   this process.

These results clearly demonstrate that LRP serves as

the major PAI-1-dependent endocytosis receptor for t-PA on

HepG2 cells, and that the 39kDa LRP receptor-associated

protein modulates this interaction.

10   *   *   *   *   *

MH_1C_1 cells rapidly catabolize the 39kDa protein as

shown in Example 11.

EXAMPLE 11

Rate of 39kDa Protein Uptake by MH_1C_1 Cells

15   The following experiment used the procedure

described in Schwartz, A.L. et al., (1982) J. Biol. Chem. 257,

4230-4237.

Rate of 39kDa Protein Uptake

Dishes containing monolayers of 10^6 MH_1C_1 cells/well were

20   cooled on ice and washed three times with cold PBSc. Ligand

uptake was initiated by the addition of prewarmed (37°C)

binding buffer containing 28 nM ^{125}I-39kDa protein, either in

the presence or absence of an excess of unlabelled 39kDa

protein. Each dish was incubated at 37°C for a period of

25   between 0 and 120 minutes. At each time point, one of the

dishes was cooled directly on ice, and the overlying media was

removed. Cell monolayers were washed three times with PBSc

and lysed in low SDS lysis buffer for the determination of

50

cell associated radioactivity. Each sample of overlying media
was precipitated with TCA as described in Example 9, and the
TCA soluble fraction was counted by γ scintillation
spectrometry.

5   FIG. 11 shows the results from a typical experiment.
The amount of cell-associated ligand increased rapidly,
reaching a plateau at approximately 40 minutes after the
transition in temperature. The amount of extracellular
degraded ligand exhibited a lag of approximately 20 minutes,
10   after which there began a linear increase which lasted the
duration of the experiment. The sum of both cell-associated
and extracellular degraded ligand also increased linearly with
a slope identical to that of extracellular degraded ligand
alone. The slopes of each of these parameters was a measure
15   of the rate of ligand uptake and degradation at 37°C and was
determined to be 52 fmol/10^6 cells/minute. This experiment
demonstrates a pattern of rapid catabolism of the 39kDa
protein by MH_1C_1 cells.

                    *   *   *   *   *

20   The following examples demonstrate that the 39kDa
protein inhibits t-PA binding to hepatic receptors.

                          EXAMPLE 12

              **Rat 39kDa Protein Can Efficiently Inhibit t-PA
                          Binding to MH_1C_1 Cells**

25   Using the procedure described in Example 3, the
^{125}I-t-PA binding analyses described below showed the kinetics
of inhibition of t-PA binding to MH_1C_1 cells by the native rat
39kDa protein. FIG. 12 shows the results of ^{125}I-t-PA binding
analyses performed in the presence of varying concentrations

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of either unlabelled t-PA or recombinant rat 39kDa protein as
competitors. Unlabelled t-PA inhibits binding of ^{125}I-t-PA with
an apparent K_i value of 8 nM (FIG. 12B). The 39kDa protein, on
the other hand, competed at a much lower concentration, with
an apparent K_i value of 0.5 nM (FIG. 12A). This experiment
demonstrates that the 39kDa protein is a strong competitor for
^{125}I-t-PA binding to LRP on the MH_1C_1 cell surface.

EXAMPLE 13

**Inhibition of ^{125}I-t-PA:PAI-1 Complex Binding to
HepG2 Cells by 39kDa Protein and Anti-LRP Antibody**

In the following ligand binding competition
experiments, it was found that PAI-1-dependent ^{125}I-t-PA binding
was also mediated via LRP. Specific binding of 3 nM ^{125}I-t-PA
was performed in the absence or presence of each of three
competitor proteins: t-PA, 39kDa protein, or bovine serum
albumin (BSA). Each competitor protein was included at
increasing concentrations up to more than 100-fold molar
excess of the radiolabelled ligand. Throughout this example,
protein concentration was determined using a Bio-Rad protein
assay with bovine serum albumin as a standard. Cell number
was determined by counting cells with a hemocytometer.

Ligand binding competition assays

Binding of the ^{125}I-labelled ligand was performed as
described above in Example 4 in the absence or presence of
various competitors at selected concentrations. In those
experiments in which both t-PA and 39kDa protein were
involved, the t-PA binding buffer was used. Nonspecific
binding was determined in the presence of 1 μM unlabelled
ligand.

52

As shown in FIG. 5A, unlabelled t-PA competed with
^{125}I-t-PA for binding with an apparent K_i of about 3 nM. The
39kDa protein also inhibited specific ^{125}I-t-PA binding to a
maximum of about 80% (FIG. 5A). The control protein BSA had
5   no effect on specific ^{125}I-t-PA binding indicating that
inhibition by the 39kDa protein was not due to a generalized
effect but was specific. These results with the 39kDa protein
suggest that the majority of the PAI-1-dependent ^{125}I-t-PA
binding on the HepG2 cells is via LRP. The observation that
10   the 39kDa protein inhibits only 80% of specific ^{125}I-t-PA
binding may indicate the presence of a minor population of
t-PA binding sites that are not accessible to or inhibited by
the 39kDa protein.

To test whether inhibition of ^{125}I-t-PA binding by the
15   39kDa protein was due to interference with formation of the
complex between ^{125}I-t-PA and endogenous PAI-1, post-binding
buffer overlying cell monolayers from the binding assays of
FIG. 5A (without any competitor protein or from each of those
with 500 nM competitor protein) was analyzed by SDS-PAGE (FIG.
20   5B). The ^{125}I-t-PA:PAI-1 complex can be seen in the absence of
excess unlabelled t-PA (lane 1), but not in the presence of
excess unlabelled t-PA (lane 2). Inclusion of excess
unlabelled 39kDa protein did not interfere with the formation
of the ^{125}I-t-PA:PAI-1 complex (lane 3). The ^{125}I-t-PA:PAI-1
25   complex observed in the presence of the 39kDa protein is
greater than that seen with ^{125}I-t-PA alone and presumably
reflects the inhibition of complex binding to HepG2 cells by
the 39kDa protein. Cell lysates corresponding to the

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overlying buffer shown in lane 1-4 were also analyzed via the
same SDS polyacylamide gel (FIG. 5B). Binding of the ^{125}I-
t-PA:PAI-1 complex to HepG2 cells is demonstrated in the cell
lysates without competitor protein (lane 5). When excess

5   unlabelled t-PA was included, however, specific binding of the
^{125}I-t-PA:PAI-1 complex was absent (lane 6). When the cell
lysate with 500 nM 39kDa protein was compared to that with ^{125}I-
t-PA alone, specific ^{125}I-t-PA:PAI-1 complex binding was reduced
by about 80% (lane 7). The control protein BSA neither

10   interfered with the complex formation (lane 4) nor inhibited
specific ^{125}I-t-PA:PAI-1 complex binding (lane 8). These
results thus demonstrate that the inhibition of ^{125}I-t-PA
binding to HepG2 cells by the 39kDa protein occurs at the
level of ^{125}I-t-PA:PAI-1 binding to LRP without interference

15   with the initial complex formation.

           Modulation of ligand binding to LRP by the 39kDa
protein is not due to competition at a single site with
various ligands since previous experiments have shown that LRP
ligands (e.g., α_2M$^\bullet$, t-PA) compete only slightly with the 39kDa

20   protein for binding to LRP. (See, Bu, G. et al., (1992) Proc.
Natl. Acad. Sci. USA 89, 7427-7431). To test whether ^{125}I-39kDa
protein binding is affected by t-PA on HepG2 cells, ligand
binding competition experiments were performed. Competitor
proteins (t-PA, 39kDa protein, or BSA) at various

25   concentrations were included in ^{125}I-39kDa protein binding. As
shown in FIG. 13, specific binding of ^{125}I-39kDa protein was
reduced only slightly (about 20%) by excess unlabelled t-PA,
whereas the binding was completely inhibited by excess

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unlabelled 39kDa protein. These results suggest that the

39kDa protein inhibits t-PA:PAI-1 complex binding indirectly

perhaps via inducing a conformational change in LRP

unfavorable to t-PA:PAI-1 complex binding. Alternatively,

5   t-PA:PAI-1 complex binding sites on HepG2 cells may account

for only a small portion of the 39kDa protein binding sites

with most of the binding sites distributed over other parts of

the LRP molecule not shared with t-PA.

To test directly whether the majority of PAI-1-

10   dependent ^{125}I-t-PA binding sites is on LRP, ligand binding

competition experiments were performed in the presence of

anti-LRP antibody. This rabbit polyclonal antibody was

generated using purified human placental LRP as antigen. The

total IgG fraction was purified and used for competition of

15   ^{125}I-t-PA binding (3 nM). Non-immune rabbit IgG was used as a

control. As shown in FIG. 14, specific binding of the ^{125}I-

t-PA:PAI-1 complex was reduced by up to 75%, while non-immune

IgG had little or no effect. The percentage of reduction on

specific ^{125}I-t-PA binding by anti-LRP antibody (i.e.

20   approximately 75%) is similar to that seen with the 39kDa

protein (see FIG. 5), further supporting the observation that

the predominant binding (~70-80%) of the ^{125}I-t-PA:PAI-1 complex

on HepG2 cells is mediated by LRP.

*   *   *   *   *

25   In the following example, it was found that the

39kDa protein effectively inhibited hepatic clearance of t-PA

in both *in vitro* and *in vivo* experiments.

EXAMPLE 14

Effect of 39kDa Protein on Hepatic Cl arance f t-PA

39kDa Protein Preparation

       The 39kDa protein was isolated from 5 liters of *E.*

5   *coli* containing the recombinant plasmid according to Example 1

and yielded the GST-39kDa protein. Following thrombin

cleavage and removal of the GST, the 39kDa protein was

approximately 95% pure as determined by SDS-PAGE and

Coomassie/silver staining. The few percent of non-39kDa

10   protein contained within these preparations were unabsorbed

fragments of GST. In order to provide a homogeneously pure

preparation of 39kDa protein, this partially purified mixture

was applied to a heparin agarose column (Sigma Chemical Co.).

Following extensive washing with 100 mM NaCl to remove

15   contaminating proteins, the 39kDa protein was eluted at 0.4 M

NaCl. This preparation resulted in the greater than 99%

homogenous preparation of 39kDa protein as determined by SDS-

PAGE and Coomassie/silver staining and shown in FIG. 15.

General Procedures

20   The following procedures were used throughout this

example.

1. Cell Culture

       Rat hepatoma MH_1C_1 cells were cultured in accordance

with the procedure of Example 3.

25   ### 2. Protein Iodination

       t-PA, 39kDa protein, GST, α1-acid glycoprotein

(orosomucoid, OR), and the asialo form of α1-acid glycoprotein

(ASOR) were iodinated with ^{125}I as described in Example 4. The

specific activity of the resultant labelled proteins was 5-10 $\mu Ci/\mu g$ of protein.

3. Protein Conc ntration Determination

Protein concentration was determined using the
Biorad protein assay with bovine serum albumin as a standard.

Ligand Binding Assays

Bioactivity was determined on this preparation of
39kDa protein by its ability to inhibit ^{125}I-t-PA to MH_1C_1 cells
as described in Example 9. Wells containing approximately 10^6
MH_1C_1 cells were washed and preincubated at 4°C with binding
buffer containing the indicated concentrations of unlabelled
t-PA or unlabelled 39kDa protein in addition to 3 nM ^{125}I-t-PA.
Following incubation for 1.5 hours at 4°C, the medium was
removed, the cells washed and the specific ^{125}I-t-PA binding was
determined based on the radioactivity measured in accordance
with the procedure of Example 4. As seen in FIG. 15, this
preparation of 39kDa protein is biologically active against
the t-PA receptor with 50% inhibition seen at <1 nM.

In vivo Clearance Determinations

In vivo clearance determinations were performed in
the following manner on 200 gm female Sprague-Dawley rats
obtained from Charles River Breeders and fed standard lab chow
ad lib. Following anesthesia with 12 mg Nembutal, the tail
vein was catheterized and a 250 μl solution containing the
test protein in normal saline was infused over 30 seconds.
Repetitive samples were obtained from the distal tail artery
into cooled microtubes containing 2 μl heparin (1:1000).
Routinely samples were collected at 30 seconds, 1, 2, 4, 6, 8

and 10 minutes. After the 10 minute peripheral sample was
collected, a central blood sample was collected by an open
cardiac puncture and the visceral organs (liver, kidney,
spleen) were rapidly removed to ice. Aliquots of the liver
5   and entire kidney and spleen were counted for radioactivity
determination. Heparinized blood samples were centrifuged to
separate plasma from blood cells and an aliquot of plasma,
generally 25 μl, was spotted onto Whatman 3M paper, dried,
precipitated in 10% trichloracetic acid and radioactivity
10   determined in a Packard Gamma Spectrophotometer.

1.   Clearance of t-PA

To demonstrate that t-PA is rapidly cleared from the
blood plasma following intravenous administration, adult 200 g
rats were anesthetized and administered 30 pmol ^{125}I-t-PA via
15   venous injection as described above. Arterial blood samples
were collected at the indicated times, separated and plasma
radioactivity determined.

As seen in FIG. 16A, following intravenous
administration to each of the eight rats of 30 pmol of ^{125}I-
20   t-PA, the plasma ^{125}I-t-PA was rapidly cleared with a $t_{1/2}$ of
approximately one minute. Less than 10% of the ^{125}I-t-PA
remained in blood plasma at 10 minutes. Analysis of the liver
at 10 minutes following the administration revealed
approximately 90% of the initial plasma radioactivity was
25   found in liver.

2.   Effect of Excess t-PA on t-PA Clearance

Intravenous administration to three rats of 400-fold
molar excess unlabelled t-PA (12 nmol) one minute prior to

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administration of the identical dose of 30 pmol ^{125}I-t-PA
resulted in a marked reduction in the plasma clearance of
^{125}I-t-PA as seen in FIG. 16A. The plasma half-life was
approximately 9-10 minutes and approximately 40% of the ^{125}I-
5   t-PA remained in the blood at 10 minutes. Analysis of the
liver at 10 minutes revealed approximately 70% of the initial
plasma radioactivity was found in liver.

        3.   Effect of 39kDa Protein on t-PA Clearance

            Intravenous administration to four rats of 250 nmol
10   of unlabelled 39kDa protein (8,000-fold molar excess) one
minute prior to administration of the identical 30 pmol ^{125}I-
t-PA also resulted in a marked reduction in the plasma
clearance of ^{125}I-t-PA as seen in FIG. 16A. Plasma half-life
was approximately 6 minutes. Approximately 30% of the ^{125}I-t-PA
15   remained in the blood at 10 minutes. Analysis of the liver at
10 minutes revealed approximately 80% of the initial plasma
radioactivity was found in liver.

        4.   Clearance of 39kDa Protein

            A series of controlled clearance experiments were
20   performed to determine the *in vivo* fate of the administered
39kDa protein. Adult 200 g rats were anesthetized and
administered 30 pmol ^{125}I-39kDa protein via venous injection.
Arterial blood samples were collected at the indicated times,
separated, and plasma radioactivity determined as described
25   above. As seen in FIG. 17, following intravenous
administration to the rat of 30 pmol of ^{125}I-39kDa protein, the
plasma ^{125}I-39kDa protein was rapidly cleared with a $t_{1/2}$ of
approximately one minute.

The following table, Table 2, shows that the
analysis of the liver, kidney and spleen at 10 minutes
revealed approximately 70% of the initial radioactivity in
liver, 7% in kidney and less than 2% in spleen. Following

5   plasma clearance of ^{125}I-39kDa protein in the rat *in vivo* as
described in FIG. 17, the liver, kidney and spleen were
harvested at 10 minutes and the radioactivity determined.
Each figure is the mean ± S.E.M.

TABLE 2

10   **ORGAN DISTRIBUTION OF ^{125}I-39kDa PROTEIN**

Preadministration		^{125}I-39kDa Protein	Liver	Kidney	Spleen
(dose)		(dose)	(% initial radioactivity)		
a.	none	30 pmol	69 ± 5	7 ± 1	2 ± 0.2
b.	12.5 nmol	30 pmol	72 ± 3	12 ± 1	2 ± 0.2
c.	50 nmol	30 pmol	25 ± 6	13 ± 2	2 ± 0.6
d.	125 nmol	30 pmol	23 ± 5	31 ± 10	2 ± 0.3

The capacity for hepatic clearance of 39kDa protein
is large, but can be saturated as seen in FIG. 17 and Table 2.
Intravenous administration of increasing doses of unlabelled

20   39kDa protein results in a decrease of the fraction of the
initial dose found in the liver at 10 minutes. Concomitantly
there is an increasing fraction of the initial dose found in
the kidney, which indicates that maximal hepatic capacity of
39kDa protein clearance can be readily achieved *in vivo*.

25   Therefore multiple dosing schedules and/or continuous infusion
of 39kDa protein should markedly reduce hepatic t-PA clearance
in vivo.

5.   Effect of Excess 39kDa Protein on 39kDa Protein Clearance

Intravenous administration of 12.5 nmol of

unlabelled 39kDa protein one minute prior to administration of

the identical 30 pmol of ^{125}I-39kDa protein resulted in a

5   prolongation of the plasma half-life to approximately three

minutes.   Analysis of liver, kidney and spleen at 10 minutes

revealed approximately 70% in liver, 12% in kidney, and less

than 2% in spleen.

Intravenous administration of 50 nmol of unlabelled

10   39kDa protein one minute prior to the identical 30 pmol of ^{125}I-

39kDa protein resulted in a further marked reduction in the

plasma clearance of the ^{125}I-39kDa protein as seen in FIG. 17

with a half-life of approximately six minutes.   Analysis of

the liver, kidney and spleen at 10 minutes revealed

15   approximately 30% liver, 15% in kidney and less than 2% in

spleen.

Intravenous administration of 125 nmol of unlabelled

39kDa protein one minute prior to administration of the

identical 30 pmol of ^{125}I-39kDa protein resulted in a further

20   marked reduction in the plasma clearance of the ^{125}I-39kDa

protein with a plasma half-life of approximately 9 minutes.

Analysis of the liver, kidney and spleen revealed

approximately 22% in liver, 33% in kidney and less than 2% in

spleen.

25   6.   Clearance of Other Proteins

Not all proteins administered to the rat

intravenously are rapidly cleared by liver.   Adult 200 g rats

were anesthetized and administered via venous injection (a) 5

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pmol ^{125}I-α1-acid glycoprotein (OR), and (b) 5 pmol ^{125}I-asialo-

α1-acid glycoprotein (ASOR). Arterial blood samples were

collected at the indicated times, separated, and plasma

radioactivity determined as described above. As seen in FIG.

5   16B, for example, α1-acid glycoprotein was cleared with a

half-time of greater than 90 minutes, whereas its asialo-

derivative was rapidly cleared with half-time of approximately

0.5 minute (see Schwartz et al., (1984) CRC Crit. Rev.

Biochem. 16, 207-233).

10   EXAMPLE 15

Effect of 39kDa Protein Fragments
on Hepatic Clearance of t-PA

The following experiment demonstrates that the

entire 39kDa protein molecule is not required for inhibition

15   of t-PA binding to the hepatic t-PA receptor. The 39kDa

protein was chemically cleaved and the individual fragments

isolated. These were then tested for inhibition of ^{125}I-t-PA

binding to receptors on rat hepatocyte cells. Throughout this

example, protein concentration was determined using a Bio-Rad

20   protein assay with bovine serum albumin as a standard.

Purified 39kDa protein was prepared in accordance

with the procedure of Example 14. In order to generate

isolated fragments of the 39kDa protein, acid hydrolysis was

performed under strict conditions. The 39kDa protein in 0.4M

25   NaCl was incubated for 80 hours at room temperature in 70%

formic acid. These acid proteolytic conditions provided for

the complete cleavage of the intact 39kDa protein at the Asp-

Pro bond at amino acid residues 114-115 and the generation of

two large fragments (~20kDa N-terminal fragment and ~28kDa C-
terminal fragment).

Following separation on SDS-PAGE, the intact 39kDa
protein, the 20kDa N-terminal fragment, and 28kDa C-terminal

5   fragment were electrophorectically transferred to PVDF paper
in accordance with the procedure described in Matsudaira, F.,
A Practical Guide to Protein and Peptide Purification for
Microsequences (1989 Academic Press, NY). The appropriate
polypeptide bands were visualized by staining representative

10   gel lanes, cut out of the PVDF paper, and eluted by incubation
for 1 hour at room temperature in 1% Triton X-100 in 50mm
Tris, pH 9. Reanalysis by SDS-PAGE demonstrates single
individual bands of the appropriate molecular weights for the
intact 39kDa protein, the 20kDa N-terminal fragment, and the

15   28kDa C-terminal fragment. Sequencing of these individual
protein species at the Washington University Microchemical
Core Facility confirmed cleavage of the intact 39kDa protein
at the Asp_{114}-Pro_{115} bond.

Thereafter the biological activity of the intact

20   39kDa protein, the 20kDa N-terminal fragment, and the 28kDa C-
terminal fragment were tested by quantitating the inhibition
of ^{125}I-t-PA binding to rat liver MH_1C_1 cells. Cells were washed
and preincubated at 4°C in binding buffer containing 0-16 nM
of the intact 39kDa protein (●), the 20kDa N-terminal fragment

25   (■), the 28kDa C-terminal fragment (▲), or PVDF-elution buffer

alone (O) as a control. 4 nM ^{125}I-t-PA was added and the
incubation continued for 2 hours at 4°C. Following removal
and washing of the unbound ^{125}I-t-PA, the amount of

radioactivity bound to the MH_1C_1 cells was determined as
described in Example 14.

As shown in FIG. 18, the intact 39kDa protein
inhibited ^{125}I-t-PA binding with 50% inhibition at approximately
1 nM and 90% inhibition at approximately 8-10 nM, consistent
with previous observations (Bu et al., (1992) Proc. Natl.
Acad. Sci. USA 89, 7427-7431; Examples 10, 12, 13, and 14
above). The 20kDa N-terminal fragment was essentially without
effect (i.e., approximately 10% inhibition at 16 nM; buffer
alone exhibited 20-30% inhibition under these identical
conditions). In marked contrast the 28kDa C-terminal fragment
was an extremely active inhibitor of ^{125}I-t-PA binding with
approximately 50% inhibition at 4 nM and 90% inhibition at 16
nM as shown in FIG. 18.

The nearly equipotent activity of the 28kDa C-
terminal fragment compared to intact 39kDa protein strongly
suggests that a small fragment of the 39kDa protein, which
could include a fragment that overlaps the dividing point
between the 20kDa N-terminal fragment and 28kDa C-terminal
fragment, or a chemically or genetically related/modified form
of the molecule, will be recognized by the t-PA hepatic
receptor and may serve as a powerful reagent in inhibiting
t-PA clearance *in vivo*.

                    *   *   *   *   *

The above description is meant to be illustrative of
the present invention, and not limiting thereof. All
explanations of the inventors' theory of the invention are for
illustrative purposes only.

SEQUENCE LISTING

(1)   GENERAL INFORMATION

    (i)   APPLICANTS: Schwartz, Alan L., Bu, Guojun

    (ii)   TITLE OF INVENTION: Methods And Compositions For Inhibition Of Hepatic Clearance Of Tissue-Type Plasminogen Activator

    (iii)   NUMBER OF SEQUENCES: 5

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    (v)   COMPUTER READABLE FORM:

        (A)   MEDIUM TYPE: Diskette, 3.50 inch, double density 720 Kb storage

        (B)   COMPUTER: IBM PC Compatible

        (C)   OPERATING SYSTEM: DOS Version 3.3

        (D)   SOFTWARE: Word Perfect 5.1

    (vi)   CURRENT APPLICATION DATA:

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(2) INFORMATION FOR SEQ ID NO: 1:

  (i)   SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 323 amino acid residues

        (B)   TYPE: amino acid

        (D)   TOPOLOGY: linear

  (ii)   MOLECULE TYPE: protein

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Tyr Ser Arg Glu Lys Asn Gln Pro Lys Pro Ser Pro Lys Arg Glu Ser
1   5   10   15
Gly Glu Glu Phe Arg Met Glu Lys Leu Asn Gln Leu Trp Glu Lys Ala
            20   25   30
Gln Arg Leu His Leu Pro Pro Val Arg Leu Ala Glu Leu His Ala Asp
        35   40   45
Leu Lys Ile Gln Glu Arg Asp Glu Leu Ala Trp Lys Lys Leu Lys Leu
    50   55   60
Asp Gly Leu Asp Glu Asp Gly Glu Lys Glu Ala Arg Leu Ile Arg Asn
65   70   75   80
Leu Asn Val Ile Leu Ala Lys Tyr Gly Leu Asp Gly Lys Lys Asp Ala
                85   90   95
Arg Gln Val Thr Ser Asn Ser Leu Ser Gly Thr Gln Glu Asp Gly Leu
            100   105   110
Asp Asp Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly
        115   120   125
Lys Phe Ser Gly Glu Glu Leu Asp Lys Leu Trp Arg Glu Phe Leu His
    130   135   140
His Lys Glu Lys Val His Glu Tyr Asn Val Leu Leu Glu Thr Leu Ser
145   150   155   160
Arg Thr Glu Glu Ile His Glu Asn Val Ile Ser Pro Ser Asp Leu Ser
            165   170   175
Asp Ile Lys Gly Ser Val Leu His Ser Arg His Thr Glu Leu Lys Glu
        180   185   190
Lys Leu Arg Ser Ile Asn Gln Gly Leu Asp Arg Leu Arg Arg Val Ser
    195   200   205
His Gln Gly Tyr Ser Thr Glu Ala Glu Phe Glu Glu Pro Arg Val Ile
    210   215   220
Asp Leu Trp Asp Leu Ala Gln Ser Ala Asn Leu Thr Asp Lys Glu Leu
225   230   235   240
Glu Ala Phe Arg Glu Glu Leu Lys His Phe Glu Ala Lys Ile Glu Lys
            245   250   255
His Asn His Tyr Gln Lys Gln Leu Glu Ile Ala His Glu Lys Leu Arg
        260   265   270
His Ala Glu Ser Val Gly Asp Gly Glu Arg Val Ser Arg Ser Arg Glu
    275   280   285
Lys His Ala Leu Leu Glu Gly Arg Thr Lys Glu Leu Gly Tyr Thr Val
    290   295   300
Lys Lys His Leu Gln Asp Leu Ser Gly Arg Ile Ser Arg Ala Arg His
305   310   315   320
Asn Glu Leu

(3)   INFORMATION FOR SEQ ID NO: 2:

    (i)   SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 209 amino acid residues

        (B)   TYPE: amino acid

        (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: protein

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly Lys Phe
1               5                   10                  15
Ser Gly Glu Glu Leu Asp Lys Leu Trp Arg Glu Phe Leu His His Lys
            20                  25                  30
Glu Lys Val His Glu Tyr Asn Val Leu Leu Glu Thr Leu Ser Arg Thr
            35                  40                  45
Glu Glu Ile His Glu Asn Val Ile Ser Pro Ser Asp Leu Ser Asp Ile
        50                  55                  60
Lys Gly Ser Val Leu His Ser Arg His Thr Glu Leu Lys Glu Lys Leu
65                  70                  75                  80
Arg Ser Ile Asn Gln Gly Leu Asp Arg Leu Arg Arg Val Ser His Gln
                85                  90                  95
Gly Tyr Ser Thr Glu Ala Glu Phe Glu Glu Pro Arg Val Ile Asp Leu
            100                 105                 110
Trp Asp Leu Ala Gln Ser Ala Asn Leu Thr Asp Lys Glu Leu Glu Ala
            115                 120                 125
Phe Arg Glu Glu Leu Lys His Phe Glu Ala Lys Ile Glu Lys His Asn
            130                 135                 140
His Tyr Gln Lys Gln Leu Glu Ile Ala His Glu Lys Leu Arg His Ala
145                 150                 155                 160
Glu Ser Val Gly Asp Gly Glu Arg Val Ser Arg Ser Arg Glu Lys His
                165                 170                 175
Ala Leu Leu Glu Gly Arg Thr Lys Glu Leu Gly Tyr Thr Val Lys Lys
                180                 185                 190
His Leu Gln Asp Leu Ser Gly Arg Ile Ser Arg Ala Arg His Asn Glu
                195                 200                 205
Leu
```

(4) INFORMATION FOR SEQ ID NO: 3:

    (i)   SEQUENCE CHARACTERISTICS:

            (A)   LENGTH: 33 bases

            (B)   TYPE: nucleic acid

            (C)   STRANDEDNESS: single

            (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (oligonucleotide)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 3:

            CCGCGTGGAT CCCCCAGGCT GGAAAAGCTG TGG   33

(5) INFORMATION FOR SEQ ID NO: 4:

    (i)   SEQUENCE CHARACTERISTICS:

            (A)   LENGTH: 35 bases

            (B)   TYPE: nucleic acid

            (C)   STRANDEDNESS: single

            (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (oligonucleotide)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 4:

            TCAATGAATT CTCAGAGTCG CTCGCCGTCG CCCAC   35

(6)   INFORMATION FOR SEQ ID NO: 5:

     (i)   SEQUENCE CHARACTERISTICS:

          (A)   LENGTH: 203 amino acid residues

          (B)   TYPE: amino acid

          (D)   TOPOLOGY: linear

     (ii)   MOLECULE TYPE: protein

     (xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly Ser Val
1               5                   10                  15
Arg Leu Thr Ser Cys Ala Arg Val Leu His Lys Glu Lys Ile His Glu
            20                  25                  30
Tyr Asn Val Leu Leu Asp Thr Leu Ser Arg Ala Glu Glu Gly Tyr Glu
            35                  40                  45
Asn Leu Leu Ser Pro Ser Asp Met Thr His Ile Lys Ser Asp Thr Leu
    50                  55                  60
Ala Ser Lys His Ser Glu Leu Lys Asp Arg Leu Arg Ser Ile Asn Gln
65                  70                  75                  80
Gly Leu Asp Arg Leu Arg Lys Val Ser His Gln Leu Arg Pro Ala Thr
            85                  90                  95
Glu Phe Glu Glu Pro Arg Val Ile Asp Leu Trp Asp Leu Ala Gln Ser
            100                 105                 110
Ala Asn Phe Thr Glu Lys Glu Leu Glu Ser Phe Arg Glu Glu Leu Lys
            115                 120                 125
His Phe Glu Ala Lys Ile Glu Lys His Asn His Tyr Gln Lys Gln Leu
    130                 135                 140
Glu Ile Ser His Gln Lys Leu Lys His Val Glu Ser Ile Gly Asp Pro
145                 150                 155                 160
Glu His Ile Ser Arg Asn Lys Glu Lys Tyr Val Leu Leu Glu Glu Lys
            165                 170                 175
Thr Lys Glu Leu Gly Tyr Lys Val Lys Lys His Leu Gln Asp Leu Ser
            180                 185                 190
Ser Arg Val Ser Arg Ala Arg His Asn Glu Leu
            195                 200
```

CLAIMS

1. A method of inhibiting the hepatic clearance of tissue-type plasminogen activator (t-PA) *in vivo* in humans comprising administering a t-PA-hepatic clearance-inhibiting amount of 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment thereof.

2. The method of claim 1, wherein the amount of 39kDa protein or fragment thereof ranges from about 60 to 6,000 mg/kg of body weight/dose.

3. The method of claim 1, wherein the fragment of the 39kDa protein is a 28kDa protein and the amount of 28kDa protein ranges from about 38 to 3,800 mg/kg of body weight/dose.

4. The method of claim 1, wherein the 39kDa protein or fragment thereof is administered 0 to 20 minutes prior to administering the t-PA.

5. The method of claim 1, wherein the hepatic-clearance of t-PA is reduced between 20 and 100%.

6. A 28kDa protein characterized by a molecular weight of 28,000 daltons on SDS-PAGE, stability to acid hydrolysis, solubility in 1% Triton X-100, and possessing biological activity against the t-PA hepatic receptor and reducing hepatic clearance of t-PA between 20 and 100%.

7. A 28kDa protein having an amino acid sequence as set forth in SEQ ID NO: 1:

```
Tyr Ser Arg Glu Lys Asn Gln Pro Lys Pro Ser Pro Lys Arg Glu Ser
1               5                   10                  15
Gly Glu Glu Phe Arg Met Glu Lys Leu Asn Gln Leu Trp Glu Lys Ala
            20                  25                  30
Gln Arg Leu His Leu Pro Pro Val Arg Leu Ala Glu Leu His Ala Asp
            35                  40                  45
Leu Lys Ile Gln Glu Arg Asp Glu Leu Ala Trp Lys Lys Leu Lys Leu
        50                  55                  60
```

```
Asp Gly Leu Asp Glu Asp Gly Glu Lys Glu Ala Arg Leu Ile Arg Asn
65              70              75                          80
Leu Asn Val Ile Leu Ala Lys Tyr Gly Leu Asp Gly Lys Lys Asp Ala
            85              90                      95
Arg Gln Val Thr Ser Asn Ser Leu Ser Gly Thr Gln Glu Asp Gly Leu
            100             105             110
Asp Asp Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly
        115             120             125
Lys Phe Ser Gly Glu Glu Leu Asp Lys Leu Trp Arg Glu Phe Leu His
    130             135             140
His Lys Glu Lys Val His Glu Tyr Asn Val Leu Leu Glu Thr Leu Ser
145             150             155             160
Arg Thr Glu Glu Ile His Glu Asn Val Ile Ser Pro Ser Asp Leu Ser
            165             170             175
Asp Ile Lys Gly Ser Val Leu His Ser Arg His Thr Glu Leu Lys Glu
            180             185             190
Lys Leu Arg Ser Ile Asn Gln Gly Leu Asp Arg Leu Arg Arg Val Ser
        195             200             205
His Gln Gly Tyr Ser Thr Glu Ala Glu Phe Glu Glu Pro Arg Val Ile
210             215             220
Asp Leu Trp Asp Leu Ala Gln Ser Ala Asn Leu Thr Asp Lys Glu Leu
225             230                     235             240
Glu Ala Phe Arg Glu Glu Leu Lys His Phe Glu Ala Lys Ile Glu Lys
            245             250             255
His Asn His Tyr Gln Lys Gln Leu Glu Ile Ala His Glu Lys Leu Arg
            260             265             270
His Ala Glu Ser Val Gly Asp Gly Glu Arg Val Ser Arg Ser Arg Glu
        275             280             285
Lys His Ala Leu Leu Glu Gly Arg Thr Lys Glu Leu Gly Tyr Thr Val
    290             295             300
Lys Lys His Leu Gln Asp Leu Ser Gly Arg Ile Ser Arg Ala Arg His
305             310             315             320
Asn Glu Leu
```

8.   A chemically synthesized gene encoding a 28kDa

protein comprising a chemically synthesized polynucleotide which

codes on expression for the amino acid sequence as set forth in SEQ

ID NO: 2:

```
Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly Lys Phe
1               5               10                      15
Ser Gly Glu Glu Leu Asp Lys Leu Trp Arg Glu Phe Leu His His Lys
            20              25              30
Glu Lys Val His Glu Tyr Asn Val Leu Leu Glu Thr Leu Ser Arg Thr
        35              40              45
Glu Glu Ile His Glu Asn Val Ile Ser Pro Ser Asp Leu Ser Asp Ile
        50              55              60
Lys Gly Ser Val Leu His Ser Arg His Thr Glu Leu Lys Glu Lys Leu
65              70              75                      80
Arg Ser Ile Asn Gln Gly Leu Asp Arg Leu Arg Arg Val Ser His Gln
            85              90                      95
Gly Tyr Ser Thr Glu Ala Glu Phe Glu Glu Pro Arg Val Ile Asp Leu
            100             105             110
```

```
Trp Asp Leu Ala Gln Ser Ala Asn Leu Thr Asp Lys Glu Leu Glu Ala
        115                     120                 125
Phe Arg Glu Glu Leu Lys His Phe Glu Ala Lys Ile Glu Lys His Asn
        130                 135                 140
His Tyr Gln Lys Gln Leu Glu Ile Ala His Glu Lys Leu Arg His Ala
145                 150                 155                 160
Glu Ser Val Gly Asp Gly Glu Arg Val Ser Arg Ser Arg Glu Lys His
                165                 170                 175
Ala Leu Leu Glu Gly Arg Thr Lys Glu Leu Gly Tyr Thr Val Lys Lys
        180                 185                 190
His Leu Gln Asp Leu Ser Gly Arg Ile Ser Arg Ala Arg His Asn Glu
        195                 200                 205
Leu
```

9.   A composition for inhibiting the hepatic
clearance of tissue-type plasminogen-activator (t-PA) *in vivo* in
humans comprising a t-PA-hepatic clearance-inhibiting amount of
39kDa protein or a t-PA-hepatic clearance inhibiting fragment
thereof.

10.   The composition of claim 9, wherein the 39kDa
protein or fragments thereof is present in a dosage amount of from
about 60 to 6,000 mg/kg of body weight/dose.

11.   The composition of claim 9, wherein the
fragment of the 39kDa protein is a 28kDa protein and is present in
a dosage amount of from about 38 to 3,800 mg/kg of body
weight/dose.

12.   In a pharmaceutical composition for a mammalian
patient containing tissue-type plasminogen activator (t-PA), the
improvement comprising said composition further including a t-PA
hepatic clearance-inhibiting amount of 39kDa protein or a t-PA
hepatic clearance-inhibiting fragment thereof.

13.   The pharmaceutical composition of claim 12,
wherein the t-PA is present in a dosage amount of from about 0.15
to 1.5 mg/kg of body weight/dose.

14.   The pharmaceutical composition of claim 12,
wherein the 39kDa protein or fragments thereof is present in a
dosage amount of from about 60 to 6,000 mg/kg of body weight/dose.

15.   The pharmaceutical composition of claim 12,
wherein the fragment of the 39kDa protein is a 28kDa protein and is
present in a dosage amount of from about 38 to 3,800 mg/kg of body
weight/dose.

16.   A composition for treating thrombolytic
diseases in a mammal comprising an effective amount of tissue-type
plasminogen activator (t-PA), and an effective amount of a t-PA
hepatic clearance-inhibiting 39kDa protein or a t-PA hepatic
clearance-inhibiting fragment thereof.

17.   The composition of claim 16, wherein the t-PA
is present in a dosage amount of from about 0.15 to 1.5 mg/kg of
body weight/dose.

18.   The composition of claim 16, wherein the 39kDa
protein or fragments thereof is present in a dosage amount of from
about 60 to 6,000 mg/kg of body weight/dose.

19.   The composition of claim 16, wherein the
fragment of the 39kDa protein is a 28kDa protein and is present in
a dosage amount of from about 38 to 3,800 mg/kg of body
weight/dose.

20.   A t-PA-hepatic clearance-inhibiting protein
characterized by binding to low density lipoprotein receptor-
related protein (LRP) and inhibiting the binding of t-PA to LRP up
to about 80%.

21.   A t-PA-hepatic clearance-inhibiting protein
comprising modified forms of the 39kDa protein and fragments

thereof which bind to LRP and reduce hepatic clearance of t-PA between 20 and 100%.

      22.   A t-PA-hepatic clearance-inhibiting protein comprising modified forms of the 28kDa protein of claim 7 which bind to LRP and reduce hepatic clearance of t-PA between 20 and 100%.

      23.   A rat protein which binds to low density lipoprotein receptor-related protein, having an amino acid sequence as set forth in SEQ ID NO: 5:

```
Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly Ser Val
 1            5                 10              Ser     15
Arg Leu Thr Ser Cys Ala Arg Val Leu His Lys Glu Lys Ile His Glu
           20                 25                 30
Tyr Asn Val Leu Leu Asp Thr Leu Ser Arg Ala Glu Glu Gly Tyr Glu
         35              40                 45
Asn Leu Leu Ser Pro Ser Asp Met Thr His Ile Lys Ser Asp Thr Leu
     50              55                 60
Ala Ser Lys His Ser Glu Leu Lys Asp Arg Leu Arg Ser Ile Asn Gln
 65              70                 75                     80
Gly Leu Asp Arg Leu Arg Lys Val Ser His Gln Leu Arg Pro Ala Thr
             85                 90                 95
Glu Phe Glu Glu Pro Arg Val Ile Asp Leu Trp Asp Leu Ala Gln Ser
             100                105                110
Ala Asn Phe Thr Glu Lys Glu Leu Glu Ser Phe Arg Glu Glu Leu Lys
         115                120                 125
His Phe Glu Ala Lys Ile Glu Lys His Asn His Tyr Gln Lys Gln Leu
     130                135                 140
Glu Ile Ser His Gln Lys Leu Lys His Val Glu Ser Ile Gly Asp Pro
145                150                 155                160
Glu His Ile Ser Arg Asn Lys Glu Lys Tyr Val Leu Leu Glu Glu Lys
             165                170                 175
Thr Lys Glu Leu Gly Tyr Lys Val Lys Lys His Leu Gln Asp Leu Ser
             180                185                190
Ser Arg Val Ser Arg Ala Arg His Asn Glu Leu
         195                200
```

      24.   A chemically synthesized gene encoding a rat protein which binds to low density lipoprotein receptor-related protein comprising a chemically synthesized polynucleotide which codes on expression for the amino acid sequence as set forth in SEQ ID NO: 5:

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```
Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly Ser Val
 1               5                   10                  15
Arg Leu Thr Ser Cys Ala Arg Val Leu His Lys Glu Lys Ile His Glu
            20                  25                  30
Tyr Asn Val Leu Leu Asp Thr Leu Ser Arg Ala Glu Glu Gly Tyr Glu
        35                  40                  45
Asn Leu Leu Ser Pro Ser Asp Met Thr His Ile Lys Ser Asp Thr Leu
    50                  55                  60
Ala Ser Lys His Ser Glu Leu Lys Asp Arg Leu Arg Ser Ile Asn Gln
65                  70                  75                      80
Gly Leu Asp Arg Leu Arg Lys Val Ser His Gln Leu Arg Pro Ala Thr
                85                  90                  95
Glu Phe Glu Glu Pro Arg Val Ile Asp Leu Trp Asp Leu Ala Gln Ser
            100                 105                 110
Ala Asn Phe Thr Glu Lys Glu Leu Glu Ser Phe Arg Glu Glu Leu Lys
            115                 120                 125
His Phe Glu Ala Lys Ile Glu Lys His Asn His Tyr Gln Lys Gln Leu
    130                 135                 140
Glu Ile Ser His Gln Lys Leu Lys His Val Glu Ser Ile Gly Asp Pro
145                 150                 155                 160
Glu His Ile Ser Arg Asn Lys Glu Lys Tyr Val Leu Leu Glu Glu Lys
            165                 170                 175
Thr Lys Glu Leu Gly Tyr Lys Val Lys Lys His Leu Gln Asp Leu Ser
            180                 185                 190
Ser Arg Val Ser Arg Ala Arg His Asn Glu Leu
            195                 200
```

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WO 94/14471   **AMENDED CLAIMS**   PCT/US93/12380

[received by the International Bureau on 22 June 1994 (22.06.94);
original claims 1-21 unchanged; original claims 22-24 amended (2 pages)]

thereof which bind to LRP and reduce hepatic clearance of t-PA between 20 and 100%.

22. A t-PA-hepatic clearance-inhibiting protein comprising modified forms of the 28kDa protein of claim 6 and fragments thereof which bind to LRP and reduce hepatic clearance of t-PA between 20 and 100%.

23. A rat 28kDa protein which binds to low density lipoprotein receptor-related protein and inhibits binding of t-PA to low density lipoprotein receptor-related protein, having an amino acid sequence as set forth in SEQ ID NO: 5:

```
Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly Ile Ser
1             5                 10                 15
Val Arg Leu Thr Ser Cys Ala Arg Val Leu His Tyr Lys Glu Lys Ile
            20                 25                 30
His Glu Tyr Asn Val Leu Leu Asp Thr Leu Ser Arg Ala Glu Glu Gly
            35                 40                 45
Tyr Glu Asn Leu Leu Ser Pro Ser Asp Met Thr His Ile Lys Ser Asp
    50                 55                 60
Thr Leu Ala Ser Lys His Ser Glu Leu Lys Asp Arg Leu Arg Ser Ile
65                 70                 75                 80
Asn Gln Gly Leu Asp Arg Leu Arg Lys Val Ser His Gln Leu Arg Pro
                85                 90                 95
Ala Thr Glu Phe Glu Glu Pro Arg Val Ile Asp Leu Trp Asp Leu Ala
            100                105                110
Gln Ser Ala Asn Phe Thr Glu Lys Glu Leu Glu Ser Phe Arg Glu Glu
            115                120                125
Leu Lys His Phe Glu Ala Lys Ile Glu Lys His Asn His Tyr Gln Lys
    130                135                140
Gln Leu Glu Ile Ser His Gln Lys Leu Lys His Val Glu Ser Ile Gly
145                150                155                160
Asp Pro Glu His Ile Ser Arg Asn Lys Glu Lys Tyr Val Leu Leu Glu
                165                170                175
Glu Lys Thr Lys Glu Leu Gly Tyr Lys Val Lys Lys His Leu Gln Asp
            180                185                190
Leu Ser Ser Arg Val Ser Arg Ala Arg His Asn Glu Leu
    195                 200
```

24. A chemically synthesized gene encoding a rat protein which binds to low density lipoprotein receptor-related protein comprising a chemically synthesized polynucleotide which codes on expression for the amino acid sequence as set forth in SEQ ID NO: 5:

```
Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly Ile Ser
1                 5                 10              15
Val Arg Leu Thr Ser Cys Ala Arg Val Leu His Tyr Lys Glu Lys Ile
            20                  25              30
His Glu Tyr Asn Val Leu Leu Asp Thr Leu Ser Arg Ala Glu Glu Gly
        35                  40              45
Tyr Glu Asn Leu Leu Ser Pro Ser Asp Met Thr His Ile Lys Ser Asp
    50                  55              60
Thr Leu Ala Ser Lys His Ser Glu Leu Lys Asp Arg Leu Arg Ser Ile
65                  70              75                  80
Asn Gln Gly Leu Asp Arg Leu Arg Lys Val Ser His Gln Leu Arg Pro
            85                  90              95
Ala Thr Glu Phe Glu Glu Pro Arg Val Ile Asp Leu Trp Asp Leu Ala
            100             105             110
Gln Ser Ala Asn Phe Thr Glu Lys Glu Leu Glu Ser Phe Arg Glu Glu
    115             120             125
Leu Lys His Phe Glu Ala Lys Ile Glu Lys His Asn His Tyr Gln Lys
    130             135             140
Gln Leu Glu Ile Ser His Gln Lys Leu Lys His Val Glu Ser Ile Gly
145             150             155             160
Asp Pro Glu His Ile Ser Arg Asn Lys Glu Lys Tyr Val Leu Leu Glu
            165             170             175
Glu Lys Thr Lys Glu Leu Gly Tyr Lys Val Lys Lys His Leu Gln Asp
            180             185             190
Leu Ser Ser Arg Val Ser Arg Ala Arg His Asn Glu Leu
            195             200
```
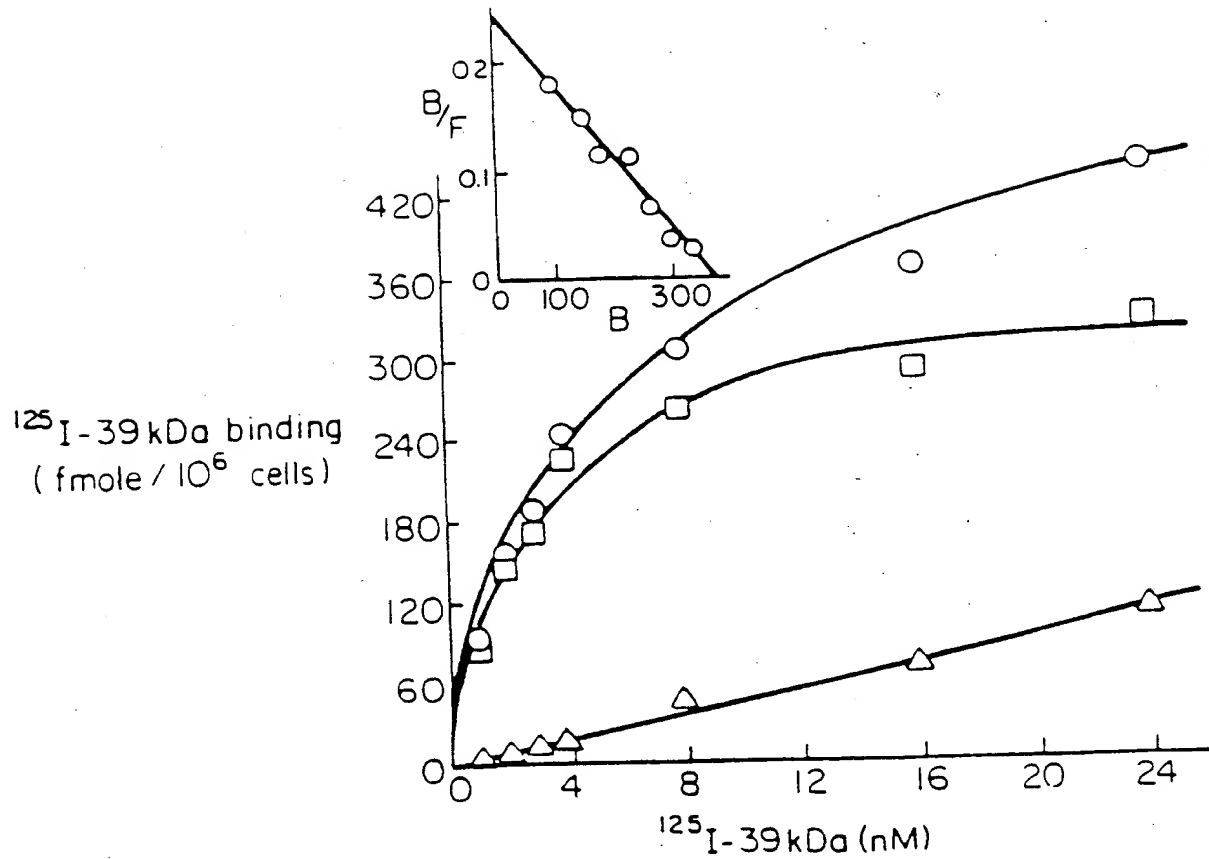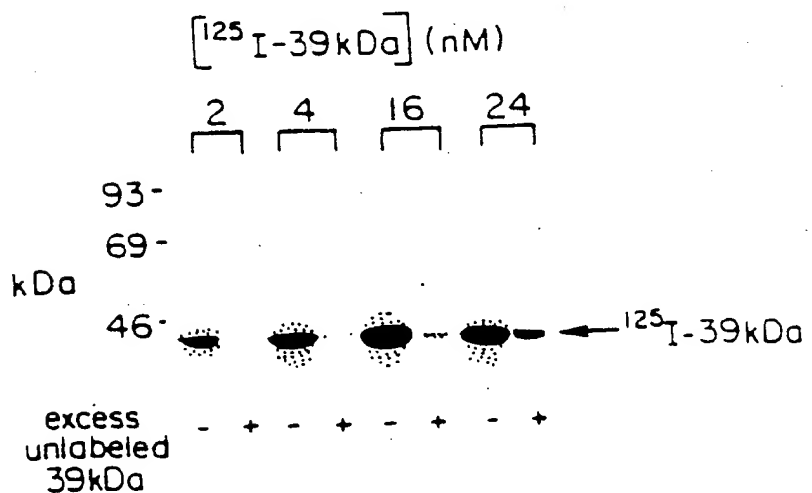
F I G. IA

F I G. IB